

Where research creates prosperity

An international comparison of research spending by companies confirms the correlation between success and research spending and raises questions about the capacity of British companies to face the future.

If a government believes it needs a policy on science and technology, there must be something seriously wrong. That subversive proposition is amply demonstrated by two of the most economically successful countries in the world: Japan and Switzerland. In each of them, government policy on science and technology is hardly distinguishable from policy on higher education. The governments concerned may agonize about particular technical issues, say air pollution and the welfare of forests (Switzerland) or the resources to be spent on space research or high-energy physics (Japan), but these are usually marginal questions. In both countries, industrial companies decide for themselves what research to do (legends about the role of Japan's Ministry of International Trade and Industry notwithstanding) and get on with it, generating tax revenues with which universities and other public services (including basic science) are supported. Who can marvel that other governments now envy that state of grace?

Even in the United States, where the rallying cry "Competitiveness!" has been fashionable since the mid-1970s, the notion that the federal government has a part to play in stimulating technological development by US industry was put into play in the first few weeks of the Clinton presidency. In reality, the new administration may have misjudged the causes of its unflattering overseas trade balance and underestimated the flexibility of the US economy, in which companies that nobody has heard of (and which may not have existed) ten years ago are now among the big players. But countries such as Britain have with good reason been glooming about the unwillingness of private companies to spend money on research for close on half a century. Elsewhere in the world, in India and Mexico, for example (see *Nature* **366**, 611–626; 1993 and **368**, 789–804; 1994 respectively), governments sponsor research themselves in the hope that companies will get the message, but may be creating perpetual dependency instead.

Shame

Britain's Department of Trade and Industry (DTI) has now performed a valuable service for all those occupied with these questions in its fourth annual compilation of company spending on research and development (see page 593). The objective is straightforward — to change company attitudes to research spending by providing information on what others do. Shame should serve the domestic purpose. In an international listing of company research spending, the biggest British spender (Glaxo, the drug company) is forty-

second in the list, the next (SmithKline-Beecham, also in pharmaceuticals) is fifty-eighth. Sixteen other European companies are in the first fifty-eight. But that is a strictly British problem. The real interest of the listings lies in what they say about the pattern of innovation in the world at large.

Numbers

DTI is properly anxious that its compilation should not be misunderstood. Its figures for spending on research and development come from sources such as published annual reports — what companies say about themselves. Definitions of what constitutes research and development vary enormously from one company to another, accounting rules from one country to another. Moreover, the inclusion of companies in DTI's listings is necessarily haphazard: public companies listed on stock exchanges are prime candidates, which means a bias towards the big and the well-established. Research-based companies still too young to have sold anything worthwhile do not figure in the statistics. Even so, the numbers make a good read.

For example, although IBM's spending fell by 13 per cent last year (to £2.99 billion worldwide), that was still comfortably ahead of the £1.75 billion spent in 1993 by Sweden's five major research companies, but uncomfortably behind the £2.34 billion spent by Fujitsu (IBM's most immediate Japanese competitor). Among 200 international companies spending more than £100 million on research in 1993, DTI finds 27 spending more than £1 billion: nine of those were based in Japan, eight in the United States, five in Germany, two in Switzerland and one each in France, Italy and the Netherlands. There were none elsewhere. Of the 200 big spenders, 81 were based in the United States and 49 in Japan. France, Britain and Germany accounted for 17, 13 and 11 respectively. The big battalions are where they would be expected, and in roughly the numbers expected.

The scale of research spending does not guarantee success; a company whose business is the manufacture of quill pens would quickly put itself out of business by spending £1 billion a year on research. But the growth of spending may be an important indicator of confidence, or of a sense of opportunity. In DTI's lists two companies doubled research spending between 1990 and 1993; they are Microsoft, the US software house, whose research spending grew from £122 million to £318 million and Sweden's Ericsson, the telephone company, which grew from £397 million to £885 million in the same period. (Spending by Alcatel-Alsthom,



the French communications giant, grew from £818 million to £1.73 billion, but mostly by mergers and acquisitions.) Others did almost as well. Glaxo, for example, chose to spend 85 per cent more on research in the same three years. The lesson to be learned is that, even during a recession, companies with a vision of where the future leads are able to help make it come about, and to their advantage.

The Japanese experience during 1990–93 should also comfort the US administration. Although Hitachi's research grew by 50 per cent between 1990 and 1992 (to a staggering £3 billion in 1993), as did that of some of the middle-sized pharmaceutical companies (Daiichi and Yamanouchi, for example), many other major companies appear to have been marking time, at least relative to past growth-rates. Sony grew by 45 per cent between 1990 and 1992 (and spent £1.41 billion on research last year), but most other electronics companies' growth appears to have been between 20 and 30 per cent, while engineering and chemicals companies in general appear almost to have stagnated over the same period, in line with expectation (see *Nature* 359, 573–582; 1992). The hesitancy of the major Japanese companies is all the more significant because Japan lacks the characteristic US network of new research-based companies, in biotechnology and electronics as well as other fields, which are now too small to qualify for DTI's listings but which may have qualified ten years from now. Indeed, the current lists include companies such as Sun Microsystems, Oracle and Genentech which, ten years ago, were simply start-up companies but which now spend £300 million, £114 million and £200 million a year on research respectively. The United States is making an impressive investment in the future, and a more adventurous investment than Japan.

Britain

What of the rest of the world? DTI's lists should well serve the domestic purpose of shaming British companies to a fuller recognition of the importance of research. By international standards, even the big spenders in Britain spend a smaller proportion of their sales turnover on research (2.29 per cent) than those in any of the other fourteen countries listed. By this yardstick, the best performers are Denmark and Canada at 14.36 per cent and 8.06 per cent respectively (but the figures are not representative, given that Denmark is represented only by Novo Nordisk and Canada by only Bell Canada and Northern Telecom). More to the point, the research budgets of the big spenders in Sweden, Germany, Switzerland and Japan are respectively 7.26 per cent, 6.80 per cent, 6.78 per cent and 5.90 per cent of their aggregate sales turnover. Even Italy (at 2.99 per cent) does better than Britain. And while these differences may be partly accounted for by differences in what the companies sell, it is mystifying that British Telecom, which likes to act the part of a latter-day AT&T, should spend only 1.76 per cent of its sales on research when its rival spends 4.57 per cent on the same cause.

Similar patterns show up even more vividly in DTI's invaluable compilation of the research budgets as a proportion of profits and of dividends paid to shareholders. In

aggregate, the companies spending more than £100 million a year on research spend as much on research as they earn as profit, but the ratio varies sharply from one country to another. In Germany, research spending by big companies is nearly five times (4.84 times) their aggregate profit, but in Sweden it is only twice as much. Japan and France are similar, with research spending to profit in the ratios 1.75 and 1.62 respectively. Switzerland and the United States come out at 80.8 per cent and 71.5 per cent, but Britain is at the other end of the scale at 28.7 per cent. Similarly, the research-based companies in Britain stand out collectively for spending less on research (74.2 per cent) than the cost of paying dividends; elsewhere the ratio of the cost of research to the annual dividend bill ranges from 1.85 (United States) to 4.25 (Switzerland) to 8.56 (Japan).

Causes

DTI has little to say about the causes of these variations, but they can be guessed at. Laggard British companies, for example, will say in self-defence that the long history of inflation and the apparently endless cycle of boom and bust has killed off the appreciation of the value of research except for companies whose interests are almost entirely in the export trade. There is substance in that argument, but it is not the whole explanation. Nor will it suffice to say that, in Britain, a greater proportion of company shares than elsewhere is held by financial institutions on behalf of individuals, pensioners and the like, for which purpose profits and dividends must be high. Yet the financial benefit of holding company shares arises in two ways, from dividends and from capital growth, each of which is denominated in the same currency. On Japan's stock markets, shareholders are content with the prospect of capital growth. And that is why, in the United States, there seems to be an endless stream of backers for the biotechnology companies forever starting up. The trouble, in Britain, is that the money-bags have ceased to believe that capital growth can outpace inflation, and so have come to regard research as an irrelevance.

Dangers

If that, or something like it, is near the truth, the outlook is not bright for Britain's shiny new science policy, whose chief objective seems to be to bring academic researchers and industrial people closer together. No doubt there will be many enlivening conversations between industrial and academic researchers, but those will not win British industry away from its belief that it must be judged by its performance in the short term, as measured by the stock exchanges and the pensions funds. Indeed, there are two obvious dangers: British industry may come to regard the future budgets of the research councils as a substitute for what it should be spending from its own resources, a perpetual subsidy of sorts. And with research training increasingly geared to applied projects, the intellectual flexibility of the products of research degree courses will not meet the next century's needs. British taxpayers (not to mention pensioners) should be hoping those dangers are avoided. DTI's quaintly named "scorecard" will help, but not enough. □

US puts large-scale AIDS vaccine trials on ice as 'premature'

Washington. Plans for large-scale clinical trials of preventive AIDS vaccines in the United States have been put on ice following the ruling last week of a key advisory panel that the candidate gp120 vaccines are not ready for such tests.

The formal decision on whether the trials should proceed rests with Anthony Fauci, director of the National Institute of Allergy and Infectious Diseases (NIAID). But immediately after his AIDS Research Advisory Committee (ARAC) came out against the trials last Friday, he said they would not go ahead.

The advisory panel's verdict overturns a recommendation in April of NIAID's Vaccine Working Group, which wanted to test two candidate vaccines and a placebo on 4,500 people at high risk of HIV infection. But Fauci denies that the decision is a setback for AIDS vaccine research.

Representatives of Genentech and Biocine, the developers of the two candidate vaccines, were devastated by the decision and angry at the way it was reached. But it has been welcomed by AIDS activists, who had doubted the value of the trials.

The decision leaves AIDS with no immediate prospects for either a preventive vaccine, a therapeutic vaccine, or a satisfactory drug therapy. It will also reinforce the recent and widely endorsed call by Bernie Fields of Harvard Medical School (see *Nature* 369, 95; 1994) for AIDS research to return to basics, and concentrate on the pathogenesis of the disease.

After eight hours of discussion, the advisory panel voted to "continue the current gp120 program and the development of other candidates currently under study", and to

"proceed with expanded clinical trial evaluation when another concept and/or compelling data from current or other studies are available".

There were 32 votes cast in favour of this wording, four abstentions and none against, enabling Fauci to claim a "unanimous" vote. But in practice the panel was deeply divided, with those supporting a large trial caving in as it became clear that they were in a minority.

Rod Hoff of NIAID's division of AIDS told the panel that the 4,500-strong trial — proposed by the working group as a half-way house to a full-scale, Phase 3 efficacy trial involving 9,000 people — would show whether the vaccines were more than 60 per cent effective or less than 30 per cent effective. But he said that a larger follow-up would be required if their effectiveness was between those levels.

Although the candidate vaccines have not been formally tested for efficacy, a number of patients have become HIV positive during safety trials, and many panel members felt that the vaccine was unlikely to be effective enough to make the 4,500-strong trial worthwhile.

The meeting also heard evidence that recruitment for the trial would be difficult, as volunteers were likely to withdraw when they were informed that the vaccine, al-

though safe, was based on part of the virus, and that some would test "false positive" for HIV for a short time during trial.

In a decisive intervention, Yung-chi Cheng of Yale University warned that a misdirected trial would damage both the future of vaccine research and public confidence. "I'm really trying to convince myself that this has some validity," he said. "[But] it doesn't have any merit on the information we have."

Working group member Susan Zolla-Pazner of New York Veterans Affairs Medical Center tried to rally support for its proposal of a 4,500-strong trial, saying that it was based on a more thorough examination of the scientific data than the 40-member advisory panel had been able to undertake.

Searching for a compromise, Warren Winkelstein of the University of California at Berkeley called for a "new mechanism" which would "move things forward without saying that we have the answer to the epidemic". But panel members argued that a large-scale trial that was not a Phase 3 efficacy trial would confuse the public, and was likely to be ineffective in meeting other declared objectives, such as establishing the correlates of immunity of the disease.

The meeting moved quickly and somewhat chaotically to issue its recommendation, effectively killing the trial. Earlier, Fauci had told the meeting that if the trial proposal was abandoned, "there may be a chilling effect on our industrial partners, although this in itself is not an acceptable rationale for proceeding".

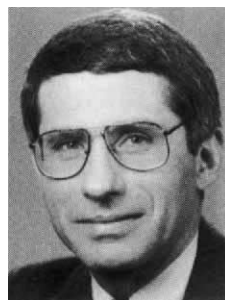
Fauci had promised himself some leeway in making a final decision after Friday's meeting. But in the event he wasted no time in endorsing the panel's advice. "I have accepted it," he said. "I have no option, and I agree with them."

Although the panel agreed on a statement stressing that their recommendation applies only to NIAID and the United States, the decision is likely to deal a hammer blow to prospects for large-scale vaccine trials in other countries.

Advocates of such trials will say that the presence of very high risk populations — for example in the sex industry in Thailand — make trials more feasible there. But opponents are now likely to argue that the vaccines rejected by the United States are being "dumped" abroad.

The United States will return to the question of a trial either when some breakthrough is made on gp120, or when alternative vaccines are ready. Fauci says this could take between one and three years.

Colin Macilwain



Fauci: welcomes the decision.

Mixed fortunes strike big spenders

London. The declining fortunes of IBM have been reflected in its drop from third to sixth place among the world's top spending companies on research and development (R&D) last year, according to figures published last week in a report sponsored by Britain's Department of Trade and Industry.

The report, *R&D Scoreboard 1993*, was prepared by an Edinburgh consulting firm, Company Reporting. The overall figures for the top 200 R&D spenders show that Japan has seven companies in the top fifteen, while the United States has four and Germany two. Britain's top R&D spending company — the pharmaceutical company Glaxo — came only 35th in the world league (see page 591).

Company spending on R&D in 1993*

\$ billion

1.	General Motors (1)	US	6.16
2.	Daimler Benz (2)	Ger	5.32
3.	Ford Motor (6)	US	5.12
4.	Hitachi (5)	Jap	4.60
5.	Siemens (4)	Ger	4.53
6.	IBM (3)	US	4.51
7.	Matsushita (7)	Jap	3.67
8.	Fujitsu (8)	Jap	3.53
9.	AT&T (9)	US	3.12
10.	Toshiba (10)	Jap	2.85
11.	NT&T (12)	Jap	2.70
12.	Alcatel-Alsthom (18)	Fr	2.61
13.	NEC (11)	Jap	2.50
14.	Asea Brown Boveri (13)	Switz	2.31
15.	Sony (14)	Jap	2.28

*(1992 ranking in brackets)

NASA puts the squeeze on low-cost missions

Washington. Concern is growing among scientists preparing to submit ideas for low-cost planetary missions to the US National Aeronautics and Space Administration (NASA) that even more stringent cost-limits have been introduced by the agency less than a month before the call for proposals is scheduled to go out.

The 'Discovery' line of planetary missions was introduced by NASA two years ago as a way of bringing down the cost of Solar System exploration, increasing the flight rate and taking advantage of new spacecraft technology. According to the programme guidelines, individual missions must cost no more than \$150 million each, take no longer than three years to develop, and be small enough to fly on a Delta rocket.

But a recent letter to the planetary research community from Wesley Huntress, NASA's associate administrator for space science, implies that missions in the \$100 million range and lower stand a better chance of being selected. It also implies that scientists should design their spacecraft for launch by cheaper, less powerful rockets.

"Given the agency projections for a decrease in space science funding over the next several years it is not possible to be confident concerning the annual funding level that might finally result from the 1996 budget process", wrote Huntress.

And he chided the planetary science community for its tendency to design Discovery proposals to "fill the bucket", with

the result that the majority of ideas for Discovery missions were at the \$150 million mark.

He estimated that future budgets for the programme would be \$100 million, and said the frequently repeated goal of one launch a year will not be possible if every proposal costs close to \$150 million. "Recent experience and studies have shown that Discovery missions with development costs less than \$100 million can produce valuable scientific data," Huntress added.

Huntress denies that the letter is intended to indicate an unofficial ceiling for proposed missions of \$100 million. He insists that NASA is not ruling out \$150 million missions, but is trying to introduce "budget flexibility" to the programme.

But many space scientists see the letter as an indication that the Discovery programme is already on shaky ground, even before the first two missions — the Near-Earth Asteroid Rendezvous (NEAR) and a small Mars lander called MESUR Pathfinder — make it to the launch pad.

Joseph Veverka of Cornell University, who has had two Discovery concepts approved by NASA for further study, called the letter "extremely worrisome", because it decreased the commitment of resources and increased the number of requirements.

Huntress also told scientists that total cost will in future be a factor in evaluating which projects get funded with low launch and mission operations costs receiving higher

marks. Many scientists proposing Discovery missions had understood from the original guidelines that a Delta rocket would be furnished at no extra cost by NASA, and that launch costs were therefore irrelevant.

Paul Spudis, a scientist with the Lunar and Planetary Institute in Houston, whose proposal for a Mercury fly-by mission was also chosen for further study, is among those whose project might be axed under the new guidelines. "If they were to down-size the vehicles, we'd be in trouble," he says.

Some researchers have been angered by Huntress' apparent implication that it is irresponsible to propose missions at or near the \$150 million mark, arguing that it was NASA, not they, who set that target in the first place. According to Veverka the original cost guidelines were "pretty reasonable" because they allowed a diversity of Solar System targets to be reached, including main-belt asteroids, Venus and Mars.

If the missions are limited to \$100 million, it will limit the number of targets says Joseph Burns, head of a National Academy of Sciences committee on planetary science that is due to publish a report on small missions later this year.

Not all scientists think that reducing the price of Discovery missions would necessarily be a bad idea. Spudis, who was on the team for the Pentagon's recent Clementine Moon mission, says he supports the idea of selecting the cheapest possible missions if they can return good science.

But changing the rules on mission cost after years of quoting the \$150 million figure amounts to a "last-minute trap door" just as scientists are about to submit their proposals. Spudis says that a budget guessing game would leave him questioning NASA's commitment to the programme.

Burns agrees that it would be better for the agency to simply come out and set a lower ceiling instead of being vague.

Huntress defends the variable pricing scheme, saying that NASA has always made it clear it was looking for a spectrum of costs in mission. But he also acknowledged that the space science community is "very, very sensitized" to news of budgetary cuts.

Many scientists in turn say they sympathize with Huntress' difficulties in trying to defend space science at a time when the agency's budget is being slashed, while the space station is being protected for political reasons. Some even believe that the cost of planetary missions will have to come down even further, to well below Discovery levels, if the programme is to survive.

But with only three missions — the Cassini Saturn probe, NEAR, and MESUR Pathfinder — currently in the funding pipeline, Discovery is, for planetary scientists, about the only game in town.

Tony Reichhardt

Scientists urge peer review on historians

Washington. The Federation of American Scientists (FAS) is urging book publishers to experiment with an equivalent of scientific peer review to avoid a repetition of the character assassination which, it claims, was carried out on physicists Robert Oppenheimer, Enrico Fermi, Leo Szilard and Niels Bohr in a book published earlier this year.

In an extensive review of the circumstances surrounding the memoir — *Special Tasks* by ex-KGB agent Pavel Sudoplatov, written with American journalists Jerrold and Leona Schecter — the FAS says that the charges in the book have been widely discredited, and attacks the attitude of New York publishers Little-Brown as "shameless" (see *Nature* 368, 779; 1994).

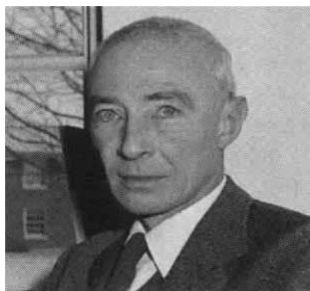
The FAS, a respected, 3,000-strong liberal pressure group whose membership includes 40 Nobel prizewinners,

calls on Gerald Levin, the chairman of Time-Warner which owns both *Little-Brown* and *Time* magazine, which published parts of the memoir, to apologize to the families of the scientists for charges which it describes as "widely denounced and unsubstantiated".

It urges publishers to check 'revelations' with expert historians, "perhaps under conditions of confidentiality to protect the news value of the disclosures." The FAS hopes publishers will be prepared to do this because they have been embarrassed by the publicity surrounding *Special Tasks*.

Sales of the book are said to have been weak after scathing reviews. But the FAS is concerned that a flood of KGB memoirs is now on the way, and that many of them are likely to contain extravagant claims in order to boost sales in the West.

C.M.



Oppenheimer: victim of new attacks.

Germany names candidate site for ITER fusion reactor

Munich. The university town of Greifswald in eastern Germany seems increasingly likely to be chosen by Germany as its proposed site for the next generation fusion reactor, the International Thermonuclear Experimental Reactor (ITER).

Last week, the Max Planck Society agreed to establish a new out-station of the Max Planck Institute for Plasma Physics (IPP) in Greifswald, which is in the state of Mecklenburg-Vorpommern (the native region of Germany's research minister, Paul Krüger), and situated on the north coast of the country.

Garching's second generation stellarator, Wendelstein 7-X, will be built there, rather than as previously planned at Garching, near Munich in the south of the country, where the IPP itself is based.

The German government's goal is to build up a service infrastructure and research environment around the new machine able to compete with other countries for the European bid for ITER, a decision due to be taken within the next couple of years.

At the same time, a department of theoretical plasma physics will be established at the University of Greifswald, with which project leaders at the IPP out-station will have joint appointments. Three new lectureships will also be created.

Wendelstein 7-X will cost DM500 million (US\$312 million) to build, half of which will be paid by the German government and 45 per cent through the European Union's fourth Framework programme. The rest will come from the state government of Mecklenburg-Vorpommern. The new machine will build on the work of its predecessor, currently operating in Garching, in determining optimal conditions for plasma burning.

Research teams at Garching have already indicated that they are prepared to move 800 km to Greifswald, accepting the pressure on the government to site important projects in the economically disadvantaged east of the country.

But the move is being made with resignation rather than enthusiasm. "We would have preferred Wendelstein 7-X built in Garching, where scientific communications would have been better and where much of the infrastructure is already in place," says a spokesman for the IPP. "Everything will have to be built from scratch at Greifswald," he adds. Indeed, Mecklenburg-Vorpommern will have to provide an extra DM120 million to establish the necessary infrastructure before building of the reactor can commence.

The decision to locate Wendelstein 7-X at Greifswald is part of the post-reunification

programme to build up the dilapidated research base of east Germany. Since becoming research minister in May last year Krüger has clearly indicated his intention of backing a German bid to host ITER at Greifswald.

One implication is that a rival bid from the Karlsruhe Nuclear Research Centre (KfK), which has already been approved by the local state government of Baden-Württemberg, is almost certain to be out of the running. This will suit the many scientists at Karlsruhe, who were worried about



ITER: Europe faces five potential sites.

losing funding for their own projects to ITER.

To increase its chance of beating bids expected from Japan, the United States and possibly Russia, Europe will, through the European Atomic Community (Euratom), propose a single site for ITER. Other candidates in Europe currently include either Studsvik or Forsmark in Sweden and Cadarache in southern France.

A final decision on the location of ITER will probably take place in about two years' time, when the engineering design phase of ITER is complete. Japan has settled on a site at Naka, and the United States is likely to choose one of several Department of Energy sites previously engaged in defence work, such as Savannah River in Georgia.

Germany has long been considered a leading candidate for Europe's next fusion reactor, following its decision in the early 1970s to support the construction of the Joint European Torus (JET) at Culham in Britain.

Alison Abbott

France in bid to avoid deadlock over LHC decision

Munich. The French government was said last week to have offered to provide an extra contribution 'in kind' thought to be worth between FF50 million (US\$8.5 million) and FF100 million above its annual contribution to the European Laboratory for Particle Physics (CERN) in order to avoid delays to the construction of the planned Large Hadron Collider (LHC).

But its offer is said to have been dependent on a resolution of a conflict between the management of CERN and the governments of Britain and Germany over two issues that were still threatening to create a deadlock when the CERN council meets on Friday (24 June) to decide whether or not to give the go-ahead to the LHC (see *Nature* 369, 509; 1994).

Britain was reported to be willing to compromise on the two countries' desire to see a change in voting procedures, which would effectively give a single country the right of veto over any inflation-linked rises during the LHC construction phase.

But Germany's research ministry, suffering from tough budget constraints, was said to be still digging in its heels both over this and a proposal that there should be a full review of the project in 1997.

The United States, Japan and the Soviet Union, are reported to be awaiting the outcome of Friday's meeting before deciding whether — and if so, at what level — to participate.

One country, however, has already indicated its willingness to do so. A draft proposal from India, spelling out the nature of potential collaboration and based on discussions with CERN officials held in Bombay in March, has been forwarded to Geneva, and India's Department of Atomic Energy is awaiting CERN's response.

The total value of an Indian contribution to the proposed LHC programme is expected to be roughly \$50 million. "But we will not be paying any cash", says a spokesman for the DAE, adding that India is proposing to provide manpower and supply hardware such as magnets and detectors for LHC, as well as "undertake any specific task assigned to us".

If the accelerator goes ahead, the monetary value of all these contributions will be assessed by CERN, and half of that value will be treated as payment toward LHC, the spokesman said. The remaining half would be used to meet the expenses of Indian scientists working in Geneva.

DAE says Indian participation in LHC is necessary not only to enable Indian scientists to take part in such experiments, but also "to establish our known expertise in high technology in the international scene". □

Fast reactor delays lead to plutonium cutbacks in Japan

Tokyo. Japan is expected to announce on Friday (24 June) plans to cut back on the production of plutonium from recycled nuclear fuel, as part of a long-awaited revision by the Atomic Energy Commission (AEC) of the country's nuclear energy policy.

The revised plans for plutonium are in part a response to growing international concerns about proliferation, and in particular to rising tensions over North Korea's suspected diversion of plutonium to the manufacture of nuclear weapons.

But the main reasons for reducing plutonium production are unrelated delays in the development of fast breeder reactors, and in the construction of facilities for the reprocessing of spent nuclear fuel within Japan.

The AEC's long-term plans, which were last revised in 1987, cover all aspects of nuclear power policy. But worldwide attention has focused in particular on plutonium. Under the existing policy, the AEC had estimated that, as a result of reprocessing fuel both within Japan and overseas, the country's supply of plutonium would rise dramatically from its current level of about 2 tonnes to 80 to 90 tonnes by 2010.

This figure far exceeds Japan's needs for its fast breeder programme, and has stimulated severe international criticism of the country's plutonium policy. Indeed, despite strong denials from the government, it has led some critics to suggest that Japan may be tempted to divert plutonium to the manufacture of nuclear weapons.

Under the revised policy, the supply of plutonium by 2010 will fall to 69 to 79 tonnes. The main reason for the reduction is that initial operations of Japan's first commercial fuel reprocessing plant, in Rokkasho in northern Japan, will be put back from 1998 to early in the next decade.

The AEC has also postponed plans for a second commercial reprocessing plant, previously due to begin operation in 2010. Instead, a decision on when to build the plant will now be made around 2010.

Government officials acknowledge that the revised policy has been influenced — even if only indirectly — by international concern over proliferation of nuclear weapons. A report on nuclear energy policy, released on 10 June by an advisory committee of the Ministry of International Trade and Industry, specifically mentions suspicion of nuclear weapon development in North Korea as a factor that has required Japan to revise its nuclear fuel cycle policy.

Two factors combine to reduce plutonium demand early in the next century.

First, Japan's failure to meet its targets for development of fast breeder reactors. Initial operation of Japan's prototype fast breeder reactor, Monju, was delayed 18 months because of technical difficulties.

Second, the electric power industry has decided to postpone building of a demonstration fast breeder reactor by several years until around 2005.

Even the new figures mean that Japan will have far more plutonium on its hands than it needs for its fast breeder programme. According to supply and demand figures in the AEC report, more than half of the plutonium produced in the period 2000 to 2010 is

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Public protests have been spectacular.

earmarked for use in ordinary light water reactors, and in a yet-to-be-built advanced thermal reactor, by mixing it with uranium.

Another factor is that commercial fast breeder reactors are not now expected to come on-line until at least 2030, rather than between 2020 and 2030, as predicted in the AEC's old policy.

The AEC report also calls for research and development on a new form of fuel reprocessing incorporating into plutonium fuel highly radioactive transuranic elements that are normally discarded as high-level waste (see *Nature* 359, 766; 1992).

This approach has the advantage of providing a way of burning up high-level waste, and also makes it more difficult to divert the plutonium to the manufacture of nuclear weapons. But there are many technical hurdles to overcome, as its much higher radioactivity means that the fuel is much harder to handle, requiring advanced robot technology.

Despite these difficulties, Japan's Science and Technology Agency, which funds the country's nuclear energy R&D, is determined to press ahead with the new technique. It plans to seek R&D funds for this purpose in its budget request for next year, due to be submitted at the end of August. The AEC report also calls for construction of a test reactor designed to experiment with this modified form of plutonium fuel.

David Swinbanks

Computer networks break new ground in agency cooperation

Tokyo. The many computer networks belonging to the various science-related government ministries and agencies in Japan seem finally to be on the verge of being merged into a comparatively seamless whole.

On Monday (20 June), Japan's prime minister Tsutomu Hata called on all government bodies to cooperate in the development of computer networks. He was responding to a report prepared by a committee of the Council of Science and Technology, Japan's leading science policy-making body, which Hata heads.

The report, entitled *How to advance present research information networks: promotion of applications*, makes repeated calls for the establishment of a "seamless" system of government computer networks.

At present, each science-related ministry and agency runs its own networks and there is very little flow of information between them. The Ministry of Education, Science and Culture, for example, runs the computer networks linking Japan's universities. Various other ministries and agencies, such as the Agency of Industrial Science and Technology, the Science and Technology Agency, the Ministry of Posts and Telecommunications, the Ministry of Agriculture, Forestry and Fisheries, and the Ministry of Health and Welfare have networks for their own researchers.

This autumn, a new inter-ministry research network will be established with gateway nodes in Tokyo, Tsukuba science city, and Osaka. This will allow full transfer of information, including electronic mail, file transfer protocol, and access to databases, between the various government networks. The new network will have a high-capacity link, operating at 2 megabits per second, with the National Science Foundation network in the United States. Links to Europe and Asia are planned for next year.

Tateo Arimoto, the director of the Science and Technology Agency's science and technology information division, says the various ministries and agencies are about to reach agreement on acceptable uses of the new network.

The network will be available for "research purposes" only. But private companies will be able to gain limited access if they have cooperative research agreements with government research organizations.

In another first for Japan, the government report on computer networks will be released on the Internet in both Japanese and English versions. It can be accessed by anonymous file transfer protocol through the Japan Information Centre of Science and Technology ([ftp:jicst.go.jp](http://jicst.go.jp)).

David Swinbanks

Court orders DNA test over chemical claims

San Francisco. A Californian court commissioner has ordered a mentally handicapped child to undergo a DNA test in what is believed to be the first use of screening technology as part of a lawsuit over workplace safety in the United States.

The court order coincides with the passage through the California state legislature of a bill designed to prohibit health insurance companies from using genetic information to identify clients with a potential risk or to raise insurance premiums.

In the case in question, lawyers defending KTI Chemicals Inc. had requested both the DNA test and a chromosome analysis of Darryl Severson. The lawsuit against the firm was filed by LeAnn Severson on her son's behalf.

Mrs Severson says her child was permanently damaged as a result of her exposure to KTI's solvents while she assembled silicon wafers. But lawyers acting for the company argue that Darryl suffers from Fragile-X syndrome.

Fragile-X syndrome is the most common cause of inherited mental retardation, and is the result of an increase in the number of CGG repeats in a gene carried on the X chromosome. The number of repeats correlates with the degree of mental retardation

and usually increases from one generation to the next. Males are more commonly affected than females, with only a third of female carriers suffering some degree of intellectual handicap themselves.

According to court documents, the Seversons are asking for \$5.6 million for Darryl's suffering and the costs of his care, education and medical treatment.

The decision to turn to DNA testing to help resolve the argument has alarmed some geneticists, who believe such uses take DNA testing far beyond its present capabilities. Paul Billings, an associate clinical professor of medicine at Stanford University, warns that although genetic information is only one component of any particular human trait or illness, society's emphasis on gene discovery and technology means that DNA testing may carry undue weight when used in courtrooms and other social settings.

Testing for Fragile-X syndrome can detect the number of CGG repeats in the gene linked to Fragile-X. If Darryl Severson has an increased number of CGG repeats, it will not prove that his retardation has a genetic origin: more than 13 per cent of boys with the Fragile-X genetic marker show no retardation at all because they have an intermediate number of repeats, and are classed as

being in the pre-mutation range. They may have learning disabilities and behavioural problems. But such a result would cast doubt on Severson's claim that toxic exposure hurt her son.

KTI argues that the chemicals in question cannot cause irreversible birth defects. The company's lawyers based their demand for a DNA test on the defendant's right to show 'alternative cause', arguing that all possible evidence should be explored to assess whether there was a chemical, genetic or some other cause for the retardation.

Once the lawyers have been provided with a sample of Darryl's blood, they may carry out additional genetic testing and search for another chromosomal explanation.

The DNA test was ordered as part of pre-trial motions. Earlier this month, lawyers for the Seversons filed a challenge to the order. Mrs Severson argues that the test is an invasion of Darryl's privacy.

Fragile-X testing has already come under fire in other arenas, reflecting concern that use of genetic testing technology may be outpacing its helpfulness. In one widely cited example, researchers have screened children in public schools in Denver, Colorado for Fragile-X syndrome, even though there is no known treatment specifically for the condition.

The case has sparked controversy because children already in special education programmes were selected for the DNA test on the basis of their behavioural and physical characteristics, potentially marking the students as genetically impaired before any DNA was examined. The director of the study, Randi Hagerman, a professor of paediatrics at the University of Colorado, is an expert witness for KTI in the Severson case.

So far, the Denver study has found five cases of Fragile-X and three cases of other sex chromosome abnormalities after screening 400 students. Hagerman claims that identifying boys with the syndrome helps to optimize the treatment and education they receive, and provides an opportunity for reassurance and genetic counselling for families. According to Hagerman, researchers are already considering proposing Fragile-X testing in the general population because the condition is so common.

Hagerman said it was a medical necessity to test for Fragile-X in cases of mental retardation where some physical characteristics of the syndrome are present. In Darryl Severson's case, she said, it was important to rule out the most common medical causes of mental retardation.

But if the test does show that Darryl has an increased number of repeats, the lawyers and their experts are likely to engage in a bitter dispute over how much it may have contributed to his condition.

Sally Lehman

Trials 'must compete for new funds'

Washington. In an unusual move, the National Cancer Institute (NCI) announced last week that the National Surgical Adjuvant Breast and Bowel Project (NSABP) will have to compete openly next year for a grant to continue running clinical trials investigating treatment methods for breast and bowel cancer.

The NSABP, based at the University of Pittsburgh, has come under fire in recent months for administrative failures in its data monitoring. At the end of March, the NCI asked the University of Pittsburgh to replace Bernard Fisher as the group's principal investigator because of its concern over the way that the project was being run.

NSABP would normally have had to submit a proposal in 1996 to continue in its role as coordinator of large-scale clinical trials. At the request of the NCI, a panel of scientific counsellors last week recommended that a new proposal should be submitted a year earlier, and in competition with other groups.

"It is unusual to have an open competition, but the NSABP has generated so much concern that it seemed needed," says Clara Bloomfield, chair of the panel and head of the department of medicine at Roswell Park Cancer Institute.

If coordinating groups such as the NSABP

are not awarded a continuation of their grant, the clinical trials they run are phased out over a year. To allay concern that the current tamoxifen breast cancer prevention trial would close if the NSABP failed in a competition, the NCI has said that it would take over the trial itself in such circumstances.

Meanwhile the controversy over the NSABP's monitoring of data continued in Congress last week at a hearing chaired by Representative John Dingell (Democrat, Michigan). Various witnesses from the NCI, Zeneca (the manufacturers of tamoxifen), and the University of Pittsburgh attempted to placate Dingell's wrath at the way the NSABP has managed its trials.

Bernard Fisher, accompanied by his lawyer, was among the witnesses. Fisher, a pioneer in the treatment of breast cancer, apologized for not acting faster once falsified data in a trial evaluating the relative effectiveness of different treatments of breast cancer had come to light.

The falsified data appeared in a trial comparing lumpectomy followed by radiation therapy with mastectomy for women with early breast cancer. A re-analysis by the NCI showed that the falsified data did not alter the finding that lumpectomy followed by radiation therapy was an effective treatment for some women. **Helen Gavaghan**

Lords fail to reverse move to ban embryo treatment

London. An amendment designed to overturn a proposed ban on the use of embryos from aborted fetuses to treat infertile women failed in Britain's House of Lords last week.

But the ban, added at the last minute by the House of Commons — with the support of Virginia Bottomley, the Secretary of State for Health — to the government's new criminal justice legislation two months ago, remains under threat, with a reworded version of the amendment likely to be tabled in July.

Last week's amendment was tabled by Lord Walton of Detchant, a former president of the General Medical Council, and would have added a wide-ranging get-out clause, retaining the general ban but allowing for the use of aborted fetuses in exceptional circumstances.

Despite receiving considerable support, the amendment was criticized for being too general, and after some debate it was withdrawn. But Walton accepted government help to reword the amendment in a more acceptable form. The new version will be debated in the House of Lords on 19 July.

The House of Commons' decision to introduce the ban had been met with widespread dismay, not least because it pre-empted recommendations to the government being prepared by the Human Fertilization and Embryo Authority (HFEA).

The HFEA is due to report next month on the response to a consultation document, which invited comments on the use of fetal embryos and other areas of research related

to *in vitro* fertilization.

According to Hugh Whittall, deputy chief executive of the HFEA, the latest developments should give the HFEA time to make preliminary comments on its findings before the Criminal Justice Bill enters its third (and final) debate in the House of Lords.

According to Walton, the ban, embodied in the amendment known as Clause 138, would cast a blight on research using fetal tissue, even though such research could lead to the prevention of miscarriages and a greater understanding of ovarian cancer.

He also highlighted the potential for the treatment of mitochondrial disease, using the cytoplasm from fetal embryos to house the nucleus of a woman's own fertilized egg thereby avoiding the damaging genes carried in the woman's own mitochondria.

He told the Lords that his amendment would allow for such crucial developments in research and in the prevention of human mitochondrial diseases to be carried out, "but only after the fullest consultation".

Progress, an organization that campaigns on behalf of research into human reproduction, said it was "encouraged" by the outcome of the debate. Marcus Pembrey, a spokesman for the group, said: "We are pleased that the government recognizes the excessive limitations imposed by Clause 138 as it stands, and the unintentional, but inevitable, blight it would have had on research."

Maggie Verrall

NCI restores funding to gene therapist seen as 'too hasty'

Washington. The board of scientific counsellors of the National Cancer Institute's (NCI)'s division of cancer treatment has agreed to restore the \$275,000 which it voted in late 1992 to withhold from a three-year contract supporting the work of Steven Rosenberg, chief of surgery at NCI.

With the additional monies approved by the board last week, the contract will now be funded at the full amount requested by Rosenberg of \$950,000 a year for three years. Rosenberg's contract is due for renewal in August, and the money will be used to pay an outside laboratory to culture cells in large numbers for clinical purposes.

At the earlier meeting, several board members had criticized Rosenberg for being too hasty in proceeding to clinical trials without the support of adequate preclinical data. As a result, the board asked him to prioritize his clinical research programme and recommended that he should receive only about two-thirds of the contract funds requested (see *Nature* 360, 399; 1992).

Specific criticism was focused on clinical trials in which tumour-infiltrating lymphocyte (TIL) cells transduced with the gene coding for tumour necrosis factor (TNF) were being used to treat patients with malignant melanomas. Some board members questioned Rosenberg's ability to get the modified TIL cells to express TNF at levels high enough to show a clinical response. Whether the data indicated that the cells were 'homing' to the tumour site, or whether TNF was being expressed at sites other than the tumour (where it could potentially be toxic to the patient) were also at issue.

In coming to their new conclusion, board members were asked to consider only new data from Rosenberg's laboratory outlining a different approach to cancer immunotherapy. Having now identified the exact nine-amino acid antigenic portion of the MART-1 (melanoma antigen recognized by T cells 1) protein, Rosenberg told the board that he plans to use this peptide to activate *in vitro* peripheral blood lymphocytes taken from a patient, and then to re-inject large numbers of these cells in the hope of obtaining a more potent anti-tumour response than that obtained with standard TIL cells.

While this new research "seems promising enough that it certainly merited the additional support", says board member Paul Sondel, a paediatric oncologist at the University of Wisconsin, it could have been appropriate to let the new work proceed "by cutting back on something else". Rosenberg says that he has no plans to scale down the TNF-TIL trials.

Diane Gershon

Double helix goes on public display

London. A reconstruction of the model of a double-helical DNA molecule — incorporating those parts of the original model that still survive since it was constructed for Francis Crick and James Watson over 40 years ago — has gone on public display at the Science Museum in London as part of a new gallery, "Health Matters".

Opening the gallery earlier this month, Watson admitted that the widely-photographed model had not served any scientific purpose, but was "something that allowed Francis to give nice talks".

Although much of the original wire model was later discarded, some of its elements were preserved at Bristol University. The model was later reconstructed at Kings College in London using these, as well as some additional replica elements.

Watson described the double helix as "a very real product of British science", and said that there was "not a better place in the world to show it off" than the Science Museum. □

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Science Museum

French minister outlines research proposals

Paris. France's National Assembly is this week scheduled to debate science for the first time since 1982. But rather than addressing a government-endorsed white paper setting out a new national strategy for research, as some had anticipated, the assembly will discuss a report from François Fillon, the minister for higher education and research, outlining his plans for the future of the research system.

The climb-down has been partly prompted by resistance from scientists to the final report of the government's six month national 'consultation' on research (see *Nature* 368, 675; 1994), which was intended to form the basis of the Assembly debate.

But following protests from the research community and a subsequent flurry of discussions with research leaders, the report has been virtually rewritten from scratch over the past eight weeks. Observers say the new version shows that Fillon appears to have listened to the views of the research community, and abandoned some of his more radical ideas.

As such the new report is likely to be given a warmer welcome by scientists. But many of those previously sceptical about the government's commitment to science have become even more so since the finance ministry froze FF900 million (US\$153 million) of the budget for research and development (R&D) last week (see *Nature* 369, 511; 1994).

In a bid to minimize the embarrassment this has caused, Fillon was expected to announce this week that he has secured an immediate reprieve of around FF220 million of the frozen money from the finance ministry. He is also said to have gained assurances from the prime minister, Edouard Balladur, that the rest of the money will be unfrozen before the end of the year.

But the National Assembly will still have relatively little to chew over. For example, it is unlikely to contest a vague promise to increase spending on French R&D from 2.4 to 2.8 per cent of the gross national product — to match the current level of the United States and Japan — by 2005.

Fillon will also propose that his ministry adopts a national research strategy to be built on reviews of strategic areas by a new permanent body, the Committee for Strategic Orientation. This will be made up of scientists, economists, industrialists and representatives from other fields. Appointed by the prime minister, it is intended to respond to requests from the ministry, and produce reviews on its own initiative.

But the concrete impact of the strategy remains unclear. Its remit will be to coordinate large multidisciplinary programmes, big equipment, applied research and technology transfer among research organizations, universities and companies. But it will

not set priorities for fundamental research, a goal which the paper describes as "illusory".

Fillon is also proposing that the National Assembly should review research strategy annually, on the eve of the vote on the research budget. This may raise the political profile of science, but risks being more of a rubber stamp than a stimulant of debate.

Many of the concrete measures expected to be proposed by Fillon this week have

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Fillon: promising university posts.

The paper calls for a new generation of *grandes programmes* (major programmes) in biotechnology, electronics, computing, new materials and other areas of economic importance. This is the long predicted consequence (see *Nature* 367, 8; 1994) of the end of the Cold War, which has reduced the political and strategic importance of the existing four big programmes, namely on space, aeronautics, telecommunications and nuclear technology, on which France spends more than half its research budget.

The biggest change is perhaps the paper's proposals to promote industrial spending on research. Until now, a few large companies have obtained the lion's share of research funding within the big programmes, but small and medium-sized companies (SMEs) will now get much more. (One "explosive" proposal, say officials, is to

already been aired before the national consultation. His ministry, for example, has already agreed to enter into five-year contracts with the research organizations in a bid to fix strategic objectives and to specify the resources allocated to achieving them.

require big companies to subcontract 20 to 40 per cent of state research funding out to SMEs).

Research funding of large companies is often little more than a state subsidy, claims one French official. By diverting money to SMEs, and setting up better evaluation procedures, the government hopes more funding will find its way into laboratories, he says, leading to an overall increase in R&D spending by industry.

Other proposals designed to encourage SMEs to do more research include collecting various funding opportunities and administrative procedures at a "single cash-point", establishing databases to help SMEs identify collaborators in public research agencies, and pressing for creation of a "Euro-NASDAQ" to increase availability of risk capital.

Fillon's most revolutionary proposal is to increase the mobility of scientists by creating a system of postdoctoral researchers. France is proud of its policy of lifetime employment, and is reluctant to create a population of researchers on short-term contracts. The Academy of Sciences will probably be asked to suggest how best to introduce the scheme.

A further incentive to mobility is a proposal to create one new university job for every transfer of staff from the research organizations (or one job for three transfers in well-staffed universities). Industrial and teaching experience would also be made a criterion for promotion.

Responding to demands from researchers, Fillon was also expected to promise to recruit new research staff at a rate of three per cent a year over the next decade in order to maintain a sensible age structure (more than half of France's current researchers will have retired by 2005). Priority areas, such as life sciences, would be allowed to recruit at higher rates.

Declan Butler

Compromise reached on bioethics bill

Paris. The French Senate and National Assembly have agreed a compromise on three bioethics bills, clearing the way for France to become the first country in the world to introduce comprehensive legislation on bioethics.

The bills were given their first reading in the National Assembly in November 1992, but last year's change in government meant that the final readings in both houses were delayed until this year (see *Nature* 367, 209; 1994). Both houses are likely to adopt the compromise by the end of this month, and the bills will then become law.

One of the most contentious issues has been the fate of supernumerary embryos,

of which there are around 11,000 in France. The Senate wanted to forbid their destruction. The compromise allows the destruction of embryos conceived before the law comes into force, but not of those conceived afterwards. The fate of the latter will be decided in five years' time, when the bioethics laws will be revised.

The Senate had also wanted to ban the pre-implantation diagnosis of embryos conceived *in vitro*, in order to avoid the risk that such techniques could be used for eugenic purposes. But it has now accepted the assembly's position that such diagnosis should be allowed in cases where parents risk conceiving a child with a serious genetic disorder.

D. B.

East Europe 'needs sustainable universities'

Bratislava. "Develop your own university systems — but learn from the mistakes we made in the West." This was the message from a public forum held last week by TERC, a body setup by the European Commission to help restructure the higher education systems of post-communist east central Europe.

Since its establishment at the Vienna-based Institute for Human Sciences in 1991, TERC — its full name is Transformation of the National Higher Education and Research Systems of Central Europe — has carried out various projects aimed at establishing links between scientists and policy makers in their countries, and at boosting communication between countries.

One such initiative of TERC's 15-strong expert committee, chaired by former European Commissioner Lord Dahrendorf and including representatives from both East and West, is the establishment of regular public forums. The second of these, held in Slovakia's capital Bratislava last weekend, addressed the extent to which the East should try to reproduce the systems of higher education and research in the West.

The consensus view was that copying is not appropriate. But it was also emphasized that the new democracies need to establish systems that are sustainable — as the old democracies have learnt to their cost.

When demand for higher education in western Europe mushroomed in the 1970s, many countries found themselves unable to cope because their higher education sector was insufficiently diversified. In Italy and Germany, for example, universities were forced to bear the strain of growing student numbers almost single handedly.

In Germany, where numbers grew from 658,000 in 1972 to 1.74 million today (discounting the new states), only in the last few years has the government taken steps to introduce alternative, more vocationally-oriented higher education establishments, such as the Fachhochschule and the Berufsakademie.

Dahrendorf warned that the economic success of east central European countries will inevitably spawn mass demand for higher education. The structures being established now should therefore be as diversified as possible, he said.

Umberto Colombo, former Italian minister for research and universities, suggested that much could be learnt from the recent experience in Italy, which is fighting to repair 40 years of damage caused by corrupt practices.

Not only has Italy faced a threefold increase in student numbers and a near doubling of the number of universities since the 1960s, but it has also suffered an eastern-style problem of rigid central control of universities.

In his brief period as minister in the last government, Colombo introduced a series of reforms which included giving financial autonomy to universities, and reducing subsidies to students.

But Colin Campbell, president of the Rockefeller Foundation in New York, said that the new democracies should not give in to pressure to introduce reforms too hastily. He warned that "some American campuses paid an enormous price" for the reforms to the university system that followed the student unrest in the 1960s.

The US reforms were necessary because the system of teaching had been too rigid, he said. But they had moved too fast, and standards at many university campuses had fallen as a result.

Does east central Europe want — or need — advice from the West? The answer was a definite yes, particularly as every country has found that its attempts to introduce workable democratic structures have been fraught with difficulties.

Compromises have been forced on science policy makers by academic communities committed in principle to democracy, but highly resistant to change. Clashes within these communities over the redistribution of power — and an instinctive reaction against

any form of state control — have both delayed reform.

A few weeks ago, for example, a new higher education bill, drafted by a committee headed by Juraj Švec, rector of Slovakia's most prestigious university, the Comenius, was thrown out by the academic community which objected strongly to his proposal that

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Czech universities are among those facing pressure to reform.

rectors — rather than faculty deans — should be the key power holders in universities.

The 1990 Universities Act in Czechoslovakia gave complete autonomy to faculties. This has made it impossible for rectors to run their universities in a way that makes it possible to drive through much-needed reforms (see *Nature* 368, 283; 1994).

Švec says his proposed bill suggested structures that are "obvious in the West", and had indeed been approved by a Council of Europe delegation visiting Slovakia last March. But others saw the bill as too much of a 'centralist model', leaving open the danger that a rector could misuse his or her power.

Objections to the proposals were so strong that the senate of Comenius university briefly considered taking steps to remove Švec from his position as rector. Ironically, however, the same senate accepted the appropriateness of the changes in a stable democracy — but not, apparently, in theirs.

"I can understand the mistrust," says Švec. "We lived in a schizophrenic society for so long we have all been infected by this mistrust." Compromises are now being sought, but it will take yet more time.

Švec, who is a member of the TERC expert committee, and former Slovak education minister Jan Pisut, both say that they welcome advice from the West. Pisut equates the present situation in east central Europe with that in western Europe in 1970, shortly after the students protests of 1968. "We should avoid the mistakes you have made since then," he told the forum last week.

Alison Abbott

Chemist to head Canadian science council

Ottawa. An inorganic chemist who arrived in Canada from England almost 30 years ago has been appointed president of the National Research Council of Canada (NRC) for a five-year term.

Arthur Carty, dean of research at the University of Waterloo, Ontario, since 1989, acknowledged last week that he plans to seek increased, stable funding for NRC and to formulate a long-term strategic plan in the context of the government's forthcoming science and technology review.

Carty also acknowledged that "there is a morale problem" at NRC resulting from the

restructuring and budget cuts of the past five years. He says that one of his first priorities will be to try to restore stability through consultations with those involved.

Carty received his doctorate from the University of Nottingham and has held visiting professorships in France and Italy. The former NRC president, Pierre Perron, resigned last August, almost a year before his five-year mandate was scheduled to end, complaining of deep budget cuts and "anaemic support of research and development" by the federal government.

David Spurgeon

European brain research

SIR — We have read with great interest the recommendations of F. Gros, G. P. Tocchini-Valentini and their committee of molecular biologists on priorities for the support of scientific research and technological development by the European Union (*Nature* 369, 11–12; 1994). We approve totally of their suggestions on the provision of European infrastructures for data libraries and for stocks of genetically modified animals. The transnational mobility of young researchers (supported by the Human Capital and Mobility programme) and the new proposal to enable young scientists to form research groups outside their country of origin must surely enhance both the excellence of European research and the cohesion of the European community.

The research programme in molecular biology suggested by the committee will have an important impact on many areas of the life sciences. The European Molecular Biology Organisation already does an excellent job in promoting many of the aims of the proposed research. We were, therefore, surprised to find that research on the brain and cognition merits such a small place in the proposed programme. This attitude contrasts sadly with that in the United States, where the 1990s are the "Decade of the Brain", and that in Japan, whose major contribution to the International Human Frontier Research Programme has enormously aided collaborative research in both neurobiology and molecular biology.

We feel that coordinated European support for specific studies on the brain in health and disease is crucial if we are not to fall behind our collaborators and competitors in the United States and Japan. Knowledge of brain function is the only way by which we can understand ourselves — how we perceive and respond to external events, how we think and how we remember. Novel physical, imaging techniques will provide important insights into these processes. The effects of ageing, addiction, and neurological and psychiatric diseases are highly damaging to our society. Rational design of drugs to combat such disorders depends crucially on research that will allow the pharmaceutical industry to enhance the health of our citizens as well as the competitiveness of European industry. An increased understanding of the molecular, synaptic and network mechanisms by which the brain stores, retrieves and analyses information is important not only in itself but also to provide a basis for the design of the next generation of computing and robotics devices.

We hope that Commissioner Ruberti can find the means to support specific projects to foster excellence in European

research and development in the neurosciences.

Jean-Pierre Changeux (*Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France*); **P. Andersen** (*Oslo, Norway*); **M. J. Berridge** (*Cambridge, UK*);

C. Blakemore (*Cambridge, UK*); **T. Bliss** (*London, UK*); **Alberto Ferrus Gamero** (*Madrid, Spain*); **T. Freund** (*Budapest, Hungary*); **R. Grantyn** (*München, Germany*); **S. Grillner** (*Stockholm, Sweden*); **K. Hepp** (*Zurich, Switzerland*); **T. Hökfelt** (*Stockholm, Sweden*); **J. Hounsgaard** (*Copenhagen, Denmark*); **H. Korn** (*Paris, France*); **R. Levi-Montalcini** (*Roma, Italy*);

R. Miles (*Paris, France*); **J.-C. Schwartz** (*Paris, France*); **W. Singer** (*Frankfurt, Germany*); **P. Strata** (*Italy*); **E. S. Vizi** (*Budapest, Hungary*); **W. Wadman** (*Amsterdam, Netherlands*); **S. Zeki** (*London, UK*)

No hitch-hikers

SIR — Bob Ward (*Nature* 368, 579; 1994) is right to raise the alarm about universities that insist on a supervisor's name being included on a paper irrespective of the supervisor's contribution, because this practice compromises the integrity of science. The reason for publishing is not to get brownie points for funding or notches on the CV gun, but to make new information public. The words 'author' and 'authority' have the same derivation and being an author means having the intellectual authority to defend the work and to take responsibility for it. That authority comes from being able to point to parts of a paper that exist only because of the author's efforts.

Claiming such authority without having it is fraudulent. For instance, part of the Statement on Scientific Practice issued by the Australian National Medical Health and Research Council, says: "Minimum requirement for authorship would be participation in conceiving and/or executing and/or interpreting at least part of the publication in a co-author's field of expertise, sufficient for him/her to take public responsibility for it. . . . 'Honorary authorship' occurs when a person is listed as an author of a publication when he/she has not participated in a substantial way in conceiving and/or executing and/or interpreting at least part of the work described in the publication. 'Honorary authorship' is an unacceptable practice."

The only room for doubt in that statement lies in interpreting "substantial", but

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E. J. Huth's five principles of authorship (*Ann. int. Med.* 104, 269–274; 1986, summarized below) help to clarify this.

(1) Each author should have participated sufficiently in the published work to take public responsibility for the content.

(2) Participation must include three steps: (i) conception or design of the work represented by the article, or analysis and interpretation of the data, or both; (ii) drafting the article or revising it for critically important content; and (iii) final approval of the version to be published.

(3) Participation solely in the collection of data (or other evidence) does not justify authorship.

(4) Each part of the content of an article critical to its main conclusions and each step in the work that led to its publication (steps (i), (ii) and (iii) in Principle (2)) must be attributable to at least one author.

(5) People who have contributed intellectually to the article but whose contributions do not justify authorship may be named and their contribution described — for example, 'advice', 'critical review of study proposal', 'data collection', 'participation in clinical trial'.

Robert Brown

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Many thanks

SIR — Science in the former Soviet Union depends and will continue to depend on the availability of information. Until 1991, there was a well developed infrastructure of libraries and express information services. But this system has almost collapsed because of the shortage of international journals and books due to the lack of hard currency.

In this situation, the help we are receiving from elsewhere is of paramount importance. Mr George Soros has done much to support libraries in the former Soviet Union, as has the German government. Scientific organizations in the United States, France and other countries are also sending us publications. We are particularly grateful for the generous response of scientists to requests for reprints. Provincial libraries and universities are even worse affected than those in Moscow, so reprints are often our only source of information.

It is not possible to write to all those who have helped and are helping us, but I should like to assure them that we read, analyse and cite their papers.

A. R. Kacimov

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If it ain't fixed, don't break it

Cecil H. Fox

Value-judgements about the need for more 'goal-directed' or 'basic' scientific research beg the question of how the publicly funded scientific enterprise works. An efficient management would start to put things right.

PEOPLE in countries where science is supported from taxes generally approve of using public money for scientific work. Unfortunately, there is rarely a fixed plan for obtaining these funds, nor does any western government have a hard and fast rule of how much money should be available. This means that support of science is dependent on the political and economic climate, creating repeated cycles aimed at making science more responsive to needs (applied science) or to wishes (basic science). Taxpayers (and most scientists) seem content to let politicians regulate the flow of money for science, but politicians are ill-prepared to deal with the everyday delivery of science, its integrity or the structural organization of the process. Mercifully, they seldom ask for an accounting in cash for scientific results.

In the United Kingdom there are elements of the government that believe science has a basic obligation to demonstrate its utility and cost-effectiveness — put simply, that all science should be applied science. In the United States, still recovering from the dark ages of the Reagan-Bush years, public funding for science is being examined from the opposing point of view. At the National Institutes of Health there is a pall of depression arising from uncertainties in the intramural research programme (see ref. 1) and a new emphasis on ill-defined 'basic' research. In Congress there is concern as to why universities are being subsidized through 'indirect costs' with money intended for scientific research. Because these funds may equal the amounts awarded to scientists for their work, radicals have even questioned why publicly supported science should be done in academic institutions or why scientists employed by private universities should require supplementary income from federal research grants.

Other factors essential to the support of science with public money are management and leadership. A frequently cited failure of applied science is the "war on cancer" in the 1970s, a concept of Richard Nixon (probably initiated by Benno Schmidt Sr). The idea was that if a problem such as cancer is carefully analysed and compartmentalized into attainable management-school-like research objectives, a solution could be expeditiously reached. A 'pie' chart showing the different programme objectives was drawn and waggishly labelled "the wheel of for-

tune". But these goals were often rapidly subverted for the convenience of scientists who intended to continue doing the research that they had always done. Worse still, managers of the programme were amateurs in the form of researchers converted to bureaucrats. Nobody got fired (indeed many are still at the same jobs), transferred or reprimanded for not accomplishing the goals set for them. These 'leaders' of the programme never appeared in the labs to assess progress. The only measure of success was in the numbers of papers produced; results were not effectively analysed and coordinated. I think the final death of the wheel of fortune occurred with the appearance of the AIDS epidemic and the rush to show that HIV was a tumour virus.

It seems reasonable to expect that scientists who become managers should have studied management in a Master of Business Administration programme before being allowed to control the expenditure of public money. The scientific skills of scientist-managers would then expedite, not impede, the work of bench scientists. In my opinion, the best-managed programme in science today, both in discovering promising young scientists and in nurturing mature ones, is the Howard Hughes Institute, a fraction of the size of the NIH. It boasts managers with experience, compassion and intelligence, and is, fortunately, immune to government politics and politicians.

Fields² has used the polio paradigm to argue that because research unrelated to polio resulted in conceptual advances leading to an effective vaccine, the future course of AIDS research should be guided by the tactic of supplying more money for basic and perhaps unrelated research. If one examines the history of polio, however, one sees that 'basic' researchers spent years passing the same strain of virus from one animal brain to another without considering the obvious enteric pathobiology of the disease. The stopgap killed vaccine (still being used) derived from this work protects only a single individual at a time and resulted in the injection of millions of children with SV40 (refs 3,4). Recognition of polio as an enteric infection, the oral vaccine (not without risk) and the resulting acquisition of herd immunity occurred only when the 'basic' discovery of cell-culture techniques was combined with 'applied' research on pathobiology of the disease. In contrast to

Fields' argument, I suggest that all science needs support for progress in any area.

HIV research is still at a stage similar to the brain transfer work in polio. Countless cultures of the same HIV strain (LAI) of virus have been used by putative basic researchers, yielding few results with any relevance to patients. Instead, an AIDS industry sprang up early. Much of the money intended for HIV research was spent on administrative and indirect costs. A lethargy has enveloped basic scientists in a hideous tautology of despair. As with polio, the recognition of HIV disease as a disease of lymphoid tissues in humans, and as such a typical lentivirus^{5,6}, has required years to achieve.

Before basic research becomes a tenet of politically correct ideological rectitude it would be wise to remember that science involves a complex equation, with modern science a détente between Aristotelian descriptive science and Baconian experimental science, where nature is 'tortured' by experiment to reveal her truth. The method of practising science is selected by individual scientists themselves according to their proclivities. Edmund Burke wrote that "men must have a certain fund of moderation to qualify them for freedom else it becomes noxious to themselves and a perfect nuisance to every body else"⁷. If science is to enjoy the support of society, a rationale may be a practical necessity. But basic research must happen under the dictates of circumstance or conscience and not as an exercise in subsidized self-indulgence. Moderation, an obligation of intellectual freedom, requires that science retains the tacit understanding that taxpaying society has agreed to support not only scientists, but creativity, for increasing knowledge and to deal with specific problems. Science is not fixed, rather it is a vital entity, basic or applied, and its lugubrious progress is more easily crippled by attempts to regularize it than not. And finally, the practice of scientific thinking with public support is a privilege, not a right. □

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Can anyons be made more tractable?

Particles that are neither fermions nor bosons, but something in between, appear to exist only fitfully, in special circumstances, but may now have been made more easily calculable.

THERE are almost as many accounts of the difference between a boson and a fermion as there are people with an interest in the question. One solution is to list them; electrons are fermions and photons are bosons, for example. Others may prefer to refer to the intrinsic quantum spin of the particles concerned; particles with half-integral spin (such as electrons) are fermions, those with zero or integral spin are bosons. Still others will choose to refer to Pauli's exclusion principle; two electrons (or two other fermions) cannot simultaneously occupy the same physical state, but the exclusion does not apply to bosons.

The exclusion principle derives from a mathematical condition that is simply put. The many-particle wavefunction for a system of, say, N fermions, will change sign when any two are interchanged, but the same operation applied to a system of bosons will yield a wavefunction that is unchanged, both in functional form and algebraic sign. This is why theoretical chemists, when constructing wave-equations for many-electron systems, are driven to write them in the form of determinants. For then the interchange of two particles will automatically bring about a change of sign; swapping any two rows (or columns) of a determinant will change the sign of the algebraic form it represents.

The practical consequences of all this are considerable. Atoms would not have electronic shell structures if the exclusion principle did not apply, nor would the electrons in a metal at low temperature fill the successive energy levels in the so-called Fermi sea up to a certain maximum, ensuring that electrons make a much smaller contribution to the specific heat of a metal than would be expected from the equipartition of energy, or on the old Dulong and Petit prediction. But liquid ^4He would not be a superfluid at low temperatures if its atoms (which have zero spin) were not bosons.

As the textbooks explain, properties such as these are best described by the "statistics", or the statistical mechanics, characteristic of fermions and bosons: fermions follow Fermi statistics, but bosons follow Bose statistics. But then the question arises whether the natural world is as sharply divided into two categories of particles as this nomenclature implies. May there not be particles that behave neither as fermions nor as bosons, but as something in between?

The question would be pointless if it could not be answered in the affirmative. Indeed, there are circumstances in which particles behave as if their properties were in

between those of fermions and bosons. The best-known are those in which electrons move in a conductor under the influence of a magnetic field, when what is known as the fractional quantum Hall effect comes into its own. (Electrons are effectively confined to two dimensions by sandwiching a microscopically thin conducting layer between two insulating layers by the techniques used in making electronic devices.)

Inevitably, there is now a name by which to call particles which are neither fermions nor bosons; *anyon* is the name used for the past decade. (The similarity with the English pronoun "anyone" is part of the joke.) But the prospect of being able to deal with them as if they were no more complicated than electrons is illusory. When electrons are confined in a narrow two-dimensional slice of conductor, for example, the properties they acquire are necessarily a function of the geometry, while the problem is a statistical problem only because there are several of them. The anyon problem is essentially a many-body problem.

What follows is not a detailed account of how the problem can now be tackled, but is rather a celebration of some work that appears to make anyons more manageable than they have been. It is relevant that people have recently become skilled at trapping small numbers of atoms in electromagnetic traps, and are robbing them of kinetic energy by suitably devised lasers. The search for direct observation of the cooperative Bose condensation expected of bosons is well under way. Using magnetic fields or other external influences to make atoms into anyons is not beyond the bounds of possibility.

The place to start is with the classical Hall effect, which is simply the observation that if a current is made to flow in some direction through a conductor, say a piece of metal foil, a magnetic field perpendicular to the plane of the conductor will induce a potential difference across the conductor in a direction transverse to the current.

The qualitative explanation is that the influence of the magnetic field is to tend to make the electrons travel in circles around its lines of flux. The quantum Hall effect, observable only at low temperatures, arises because low-energy electrons in a magnetic field should be confined to orbits in which their momentum is quantized. But observations (notably by the German physicist K. von Klitzing, who won a Nobel Prize a decade ago for his pains) show that there are many more quantized Hall voltages than

expected (whence the fractionality). These are anyons made manifest.

The impediments to the solution of this problem are easily imagined. The quantized motion of a single electron in an external magnetic field is not straightforward, the application of the exclusion principle is something else again. But three years ago, F. D. M. Haldane from Princeton University described a "generalization of the Pauli principle" that seemed to offer a way of dealing with anyons in a coherent fashion (*Phys. Rev. Lett.* **67**, 937; 1991). The trouble was that the argument involved counting the number of dimensions in a Hilbert space in which the state of the quantum system could be represented, hardly common practice.

But it makes sense. A Hilbert space is most simply thought of as one in which each axis is one of a set of orthogonal (and, in general, complex) functions, which may, for example, be the eigenfunctions of a Schrödinger equation, each corresponding to a quantum state. Then any point in the Hilbert space will represent a mixture or superposition of quantum states. In most applications, the Hilbert space has an infinite number of dimensions. But for electrons moving in a two-dimensional piece of conductor with finite dimensions (and ignoring the complication of excitation into states of higher energy), the Hilbert space describing the motion of a single particle will have finite dimensions.

To describe N particles, of course, extra dimensions will be necessary, in the extreme case of fermions, as many single-particle spaces as there are particles. Haldane's generalization of the exclusion principle was simply a statement of how the size of the single-particle Hilbert space would change as extra particles were added.

M. V. N. Murthy and R. Shankar from the University of Madras have now taken the argument an important step further (*Phys. Rev. Lett.* **72**, 3629–3633; 1994) by showing that the single-particle Hilbert space need not necessarily have a finite number of dimensions. Among other things, they are able to calculate the single-particle partition function of a system of anyons, from which all thermodynamic properties should flow. As people do, the authors have calculated a few simple systems to show that their argument holds water. But the prize that lies ahead is that it may be useable in tackling the outstanding problem of the interacting spins of ferromagnetic materials, perhaps even of high- T_c superconductors.

John Maddox

Cycling splicing factors

David L. Spector

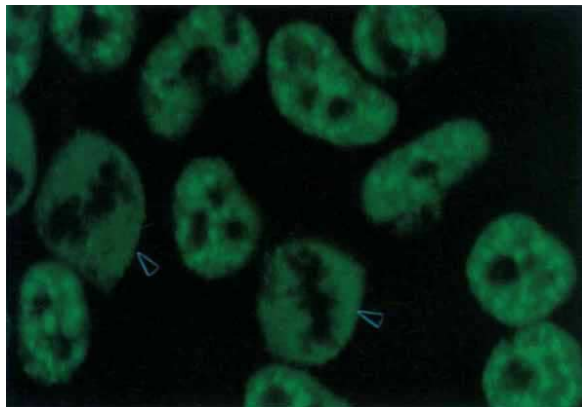
It is 25 years since it was shown biochemically that small nuclear ribonucleic acids (snRNAs) redistribute to daughter cells at each cell division¹. On page 678 of this issue², Gui *et al.* describe the identification of a kinase that may be responsible for the redistribution of splicing factors, including snRNPs which contain snRNAs, as cells enter mitosis. The same kinase inhibits splicing *in vitro*, so it may also participate in modulating pre-messenger RNA splicing activity.

During interphase, snRNPs (small nuclear ribonucleoprotein particles) and other pre-mRNA splicing factors are organized in a speckled pattern in the nucleus of mammalian cells. This pattern is composed of both perichromatin fibrils and interchromatin granule clusters (see ref. 3 for review). It has been proposed that the perichromatin fibrils represent nascent transcripts and the interchromatin granule clusters are storage or reassembly sites (or both) for splicing factors (see ref. 4 for review). Recent results have led to the suggestion that splicing factors are recruited from the storage/reassembly sites to the sites of active transcription of intron-containing genes⁵. On entry into mitosis, the speckled pattern breaks up as the splicing factors become redistributed throughout the cytoplasm. Late in mitosis the pattern begins to reform, and by the G1 phase of the cell cycle each daughter cell once again exhibits a speckled distribution of splicing factors (see ref. 3).

Although several studies have provided a glimpse of many of the players in this orchestra, the conductor has remained elusive. Enter Gui *et al.* — while examining the possibility that p34^{cdc2} is the conductor, they have come up with a new virtuoso, SRPK1, a kinase whose activity is cell-cycle regulated. This kinase phosphorylates a family of proteins, referred to as SR proteins (ref. 6), as well as other splicing factors that contain a domain rich in arginine/serine (RS) repeats. The SR proteins are highly conserved from plants to humans (see ref. 7 for review), their RS domain being responsible for targeting them to nuclear speckles⁸, and for protein-protein interactions among family members^{9,10}. SR proteins can restore splicing activity to S100 extracts, and they are central to the process of constitutive or alternative pre-mRNA splicing (see ref. 11 for review).

Gui *et al.* have fractionated extracts from mitotic cells and find that the activity of SRPK1 is distinct from that of p34^{cdc2}

kinase. The newly identified kinase induces a redistribution of SR proteins and snRNPs when added in purified form to permeabilized cells, but has no apparent effect on several other nuclear constituents examined. It is not yet known if the action of the kinase results in the specific release of SR proteins and snRNPs from the interchromatin granule clusters, or if it causes a complete dissociation of these



Pre-mRNA splicing factors are organized in a speckled pattern in interphase nuclei. Upon entry into mitosis (arrowheads) these factors are redistributed diffusely throughout the cytoplasm. A kinase, SRPK1, may be responsible for this redistribution. (Photo by David L. Spector.)

clusters. Also, more experiments will be required to determine if the kinase has additional substrates, and if the phosphorylation state of a particular substrate is directly responsible for the observed redistribution. Because SRPK1 activity was also evident in extracts from unsynchronized cells, Gui *et al.* raise the possibility that it may also be involved in the regulation of the activity of splicing factors during interphase. It is tantalizing to suggest that changes in the phosphorylation state of these factors may be the trigger for the observed recruitment of splicing factors between storage/reassembly sites and sites of active transcription⁵.

There is evidence that phosphorylation and dephosphorylation have a part in the splicing reaction. For example, last year Wopmann *et al.*¹² identified a kinase activity that phosphorylates serines in the RS domains of the U1 70K protein and SF2/ASF, a member of the SR family. The substrate specificity of SRPK1 raises the possibility that it is the kinase responsible for this activity. Phosphatases have been implicated in both catalytic steps of pre-mRNA splicing *in vitro*, but not in spliceosome assembly^{13,14}. The study of Gui *et al.* is the first to implicate phosphorylation in the cell-cycle regulation and organization of splicing factors. Because kinases usually work in concert with protein phosphatases, it is likely that one or more phosphatases will also be involved in this process.

tases, it is likely that one or more phosphatases will also be involved in this process.

In addition to its role in reorganizing splicing factors, Gui *et al.* found that addition of SRPK1 to a splicing reaction *in vitro* resulted in a dose-dependent inhibition of pre-mRNA splicing. It will be interesting to determine if the constitutive and alternative splicing activities of SR proteins differ when they are purified from interphase or mitotic cells.

Sequence analysis has revealed that SRPK1 is related to both the fission yeast kinase Dsk1 (ref. 15) and a hypothetical kinase in *Caenorhabditis elegans*.

Although the *dsk1* gene affects chromosome segregation during mitosis, it is not essential for viability; overexpression, however, results in a delay in progression from G2 to mitosis. Although Dsk1 and SRPK1 share homology, they may have different roles in the cell — SR proteins have not yet been identified in yeast and, furthermore, it is unclear if all splicing factors are organized in a speckled pattern in yeast nuclei (see ref. 3 for review). The sequence of SRPK1 includes two stretches of basic amino acids, which may function as nuclear targeting signals, but it will be essential to determine the sub-localization of the kinase in the nucleus (is it in the speckles?) and whether or not its distribution is regulated by the cell cycle.

The findings of Gui *et al.* bring to the forefront the problems of how factors may be maintained in particular subnuclear domains, and how their redistribution is orchestrated. The whole story is not yet in. But the prologue is very thought-provoking, and we can expect exciting developments over the next few years. □

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Memories are made of this?

Polly Matzinger

PAPERS on pages 648 and 652 of this issue^{1,2}, together with other work³, highlight a controversy about the way the immune system remembers past encounters with environmental pathogens. The debate centres on cellular lifespans. The old view is that newly made B and T lymphocytes of the immune system are short-lived, dying within a few weeks to make room for new cells. However, if they see an antigen, they divide to make armies of effector cells to combat the infection and of long-lived memory cells which can remain quiescent for years. The newer view, sparked by Gray and colleagues, is that memory cells are not intrinsically longer-lived than virgin cells but that memory depends instead on the persistence of antigen.

The question is about the dilemma facing a homeostatic immune system. Like librarians with limited shelf space, who must decide whether to keep old, seldom-used texts or replace them with more current titles, the immune system must either keep memory T cells against rare antigens, though they may never again be needed, or make space for expansion of lymphocytes against pathogens in the immediate environment. Fortunately there is a way in which it could do both, using one mechanism for short-term and another for long-term memory.

Short-term memory

Short-term memory is the job of follicular dendritic cells (FDCs), which bind antigens (via antibody) and slowly release them. During a response to a virus, for example, specific T and B cells are activated, multiply, become effectors, make antibody and clear the virus. Viral antigens are then slowly released by local FDCs and captured by virus-specific B cells, which process and present them to memory T cells, each encounter acting to keep the response going for months. In this way the immune system can prolong the response in case the pathogen is a fairly common one for which constant immunity is necessary. Subsequent infections will boost the responding cells and replenish the store of retained antigen. If the virus is rare, however, the response will eventually wane as the store of FDC-bound antigen is used up. This may be why many vaccines give long-lasting but not permanent protection.

Supporting this view are studies in which memory T and/or B cells were transferred from an immunized animal to naive recipients, with or without a new dose of antigen⁴⁻⁷. If memory resides in long-lived cells, the presence or absence of antigen should be irrelevant, but in

these studies memory cells transferred without antigen lost their function after a couple of months. The memory loss was documented for B and T helper and killer cells, in rats and mice, for four different antigens, with irradiated or unirradiated recipients: in each case, memory persisted if antigen was given at the time of transfer. The authors of these papers concluded that memory is not maintained by long-lived cells but by stimulation with antigen, perhaps the antigen retained by FDCs.

A problem with this explanation was that antigens picked up from FDCs should, by current dogma, be coupled efficiently to class II but not class I molecules of the major histocompatibility complex (MHC), and could therefore re-stimulate B cells or T helpers (which, respectively, see native antigens or peptides bound to MHC class II molecules) but not killer T cells, which see peptides bound to MHC class I. It is this problem that prompted the latest studies¹⁻³.

Using influenza virus, Mullbacher³ transferred memory cells into naive or previously primed recipients (reasoning that they should contain FDC-bound antigen), found long-lasting killers in both groups and concluded that memory lasts without antigen. Lau *et al.*¹, transferred memory cells against lymphocytic choriomeningitis virus (LCMV) into naive mice, transferred again after 18 months and found that the frequency of killers hovered around 1 in 1,000 for 26 months, longer than the average mouse's lifespan. In a reality check, they infected mice with a virulent LCMV and found that the recipients were completely protected. Failing by different methods to find any transferred LCMV antigen, they concluded that memory does not require antigen persistence. Hou *et al.*², reasoning that even the best methods might not detect residual Sendai virus, used MHC class I-deficient recipients. Although there should have been no MHC/peptide complexes available, the frequency of memory killers remained steady for almost six months. Again, the authors concluded that memory persists without antigen.

The most commonly cited studies in support for long-lived memory cells are those showing that ankylosing spondylitis patients and war victims still have lymphocytes containing abnormal di- and acentric chromosomes 40 years after radiation. Such cells should die after mitosis, so it has been argued that these are the non-dividing, memory cells. A lesson from *Drosophila* genetics, however, suggests that this might be a red herring. Because radiation can induce chromosomal inversions, and recombination within the in-

verted segment produces di- and acentric, the abnormal cells in the blood might have been 1 day rather than 40 years old.

Nevertheless, the famous case of the Farøe Islands shows how long memory can last. In 1781, this tiny isolated island group, visited only rarely by Swedish trading ships, was hit by an epidemic of measles which decimated the population. In 1846, measles struck again, brought by a contaminated sailing vessel, and this time the King sent his royal surgeon to the islands. His report⁸, a model of careful analysis and rational thought, noted that people older than 64 did not become ill and suggested that they were protected by their exposure to the 1781 epidemic. The point here is that neither their children nor close relatives were protected, showing that the islands had seen no measles for 64 years. What accomplished this feat of immunological memory? A single infection is unlikely to supply the FDCs with enough antigen to last for 64 years!

Long-term memory

One solution to the experimental contradictions as well as to the 'shelf space' problem comes from Selin *et al.*⁹, who analysed T-cell responses to pichinde and vaccinia viruses in mice that had previously been immunized with LCMV. Although the viruses do not cross-react serologically, and although primary killers raised against one do not normally lyse targets infected with the others, the cross-reactions for re-stimulation of memory were substantial. For example, less than 1% of primary killers to pichinde react with LCMV; but in mice previously primed to LCMV, 23% of killers raised against pichinde had dual specificity for both viruses. Thus the primary response to pichinde was replete with memory cells to LCMV. Such competition between memory and naive cells has long been known for the B-cell response to flu, where antibodies to a new variant are overwhelmingly directed to antigens shared with a previously seen variant. Because T cells see short peptides of 8-13 amino acids and they can cross-react to variants of the original peptide, as well as to unrelated MHC/peptide complexes, Selin *et al.* note that T-cell cross-reactions need not be limited to related pathogens.

This is the first important feature for cross-reactive memory. The second is that memory T cells are exposed to a world of antigens that are not available to virgin T cells because, unlike virgin cells, they can respond to antigens presented on B cells, and B cells, using their surface immunoglobulin, are exceedingly efficient at capturing low-density antigens. As an individual ages, because of the two features of cross-reactivity and response to B cells, the system will become dominated by memory cells that also respond to current environmental antigens. In this way, the

immune system can maintain memory to very old and rare pathogens while responding to new ones. (This may also be why children who have had their thymuses removed early in life can nevertheless respond to most common pathogens.)

Because the length of memory to any particular antigen will depend on its relationship to the current environment, it may not be surprising that investigators working with different systems have come up with different results. Gray and I (ref. 6) used haemocyanin from deep-sea keyhole limpets and the male antigen, H-Y, both of them simple antigens to which most (female) rodents are not exposed; our transferred T cells lost their memory. By contrast, the complex structures of flu, LCMV and vaccinia offer a greater probability of cross-reactions with environmental antigens. In fact there are hints in the papers of Lau *et al.*¹ and Hou *et al.*² that cross-reactions may well have been involved. If memory T cells were quiescently long-lived, their numbers should be steady but their proportional representation should drop in an immune system responding to the environment. But the frequencies of the putatively quiescent memory T cells remained the same for the life of the experiments.

Although Hou *et al.*² made a valiant attempt to test for cross-reactions by using β_2 -microglobulin-knockout mice as recipients, their set-up was not perfect. First, these mice do express low amounts of MHC class I molecules, perhaps enough to boost memory T cells. Second, 30% of the transferred (MHC class I-positive) inoculum consisted of non-T, non-B cells. Spleen is a good source of stem cells, so the recipient animals should have become populated with enough MHC class I-positive antigen-presenting and B cells to allow for cross-reactive memory.

All in all, then, I favour the view that the immune system keeps a library of FDC-bound antigen to maintain short-term memory to common environmental pathogens, and that it uses cross-reactions to maintain long-term memory and ensure that memory cells specific for rarer pathogens are relevant to the current environment. But the debate will continue — not least because the rational design of vaccines depends on the outcome. □

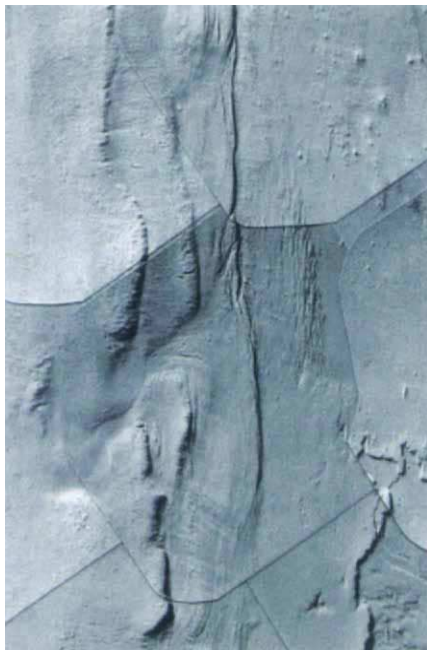
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Stirring times for Mars

Sean C. Solomon

THE theory of plate tectonics accounts admirably for the current motions of the Earth's tectonic plates, which separate at mid-ocean ridges, converge at deep-sea trenches and active mountain belts, and slip horizontally past one another along great fault zones. But the importance of plate tectonics early in Earth's history is uncertain, and the conditions favouring its development still more so. The docu-



Viking Orbiter image of a portion of the Gordii Dorsum escarpment on Mars (courtesy of J. R. Zimbelman). The image, 47 km wide, is centred near 3° N, 143° N (northwest is up). Forsythe and Zimbelman¹¹ have argued, on the basis of apparent 'push-up' structures and other similarities to terrestrial fault zones and laboratory analogues, that this feature is a strike-slip fault recording at least 30 km of left-lateral motion. Gordii Dorsum is one of a family of subparallel, northwest-striking ridges in an equatorial region west of Tharsis Montes. In Sleep's plate tectonic reconstruction³, Gordii Dorsum is interpreted as a plate breakup margin, and the ridges to the west are interpreted as analogues to terrestrial abyssal hills.

mentation of plate tectonics on another planetary body would be an important step towards a deeper understanding.

The smaller terrestrial planets, the Moon and Mercury, are characterized by generally ancient, heavily cratered crusts showing no evidence in their landforms even for past episodes of plate tectonics. Venus, the planet most similar to the Earth in mass and radius, has an abundance of diverse tectonic features and an average surface age of only a few hundred million years, but there is no global system

of tectonic plates¹, and whether episodes of plate tectonics existed in the past² is an open issue. The latest wrinkle on this topic is a proposal by Sleep³ that plate tectonics might once have operated on Mars.

When the first global views of Mars were obtained by Mariner 9 in the early 1970s, there were two paradigms for the evolution of a rocky planet. The first was terrestrial plate tectonics; the second was the lunar pattern, in which the development of a thick, low-density crust during the differentiation of an early global magma ocean made surface plates too buoyant to subduct into the interior and thus forever precluded plate tectonics⁴. Although a few analogies were drawn at that time between martian and terrestrial features⁵, most of those in the field interpreted the generally high densities of impact craters and the absence of modern plate tectonic features as evidence that Mars, intermediate in size between the Earth and the Moon, followed the lunar paradigm.

Two chief features of martian geology, however, have proved difficult to explain in this way: the 'crustal dichotomy' and the concentration of younger volcanism into a few large provinces. The first of these refers to the contrast between the younger northern lowlands and the more heavily cratered uplands. Gravity anomaly measurements indicate that the 3-km greater elevation of the uplands is approximately balanced by a crust that is about 20 km thicker than in the lowlands⁶. This difference in age and thickness has been variously ascribed to a giant impact basin centred on the northern lowlands⁷, several smaller overlapping impact basins⁸ or thinning of the crust by mantle convection⁹. Sleep now suggests that the northern lowlands are the result of older crust having been subducted and replaced by younger, thinner crust formed at one or more spreading centres.

Although volcanic plains are widespread on Mars, the youngest volcanism was concentrated in a few large regions, most notably the Tharsis province. The large volcanic flux and associated deformation of the Tharsis province have been ascribed to a variety of causes¹⁰, but no ready explanation has previously been found for why the Tharsis region is located astride a portion of the dichotomy boundary. Sleep argues that the Tharsis Montes volcanoes may have begun as volcanic centres along a martian subduction zone.

The principles governing subduction and the generation of sea-floor features on Earth are well enough understood that scaling to lower gravity can set limits on some of the characteristics of plate tect-

onics on Mars. For instance, the lack of obvious fossil scarps along martian fracture zones permits a lower bound on spreading rate (for a given plate-boundary geometry), as does the observation that the spreading rate must exceed that of the transition from rough to smooth axial topography (Sleep estimates that the dividing line would occur at a full spreading rate of about 30 millimetres a year on Mars). The thickness of crust generated at a spreading centre, for a given mantle potential temperature, scales inversely with gravity⁴, as does the change of crustal thickness with mantle temperature. Sleep conjectures that if the crust of the southern uplands was generated by plate tectonics as well, the younger and thinner lowland crust formed after the mantle had cooled by about 100 °C. The minimum age for subduction scales inversely as the square of gravity, so he suggests that reorganizations in plate geometry would be expected as spreading centres approached martian trenches.

In a move guaranteed to send many workers in martian geology scurrying back to their benches, Sleep has offered a specific plate reconstruction for the formation of the northern lowlands. According to that reconstruction, the high-standing linear features Phlegra Montes and Scandia Colles are transform fault tracks or traces of plate triple junction, the Gordii Dorsum escarpment¹¹ is a

plate breakup margin (see photograph), and the volcanoes Olympus Mons and Alba Patera sit astride abandoned ridge-axis segments that remained magmatically active after spreading ceased. Further, his proposed timing for the plate tectonic formation of the lowland crust would rule out earlier proposals⁷⁻⁹ that rim remnants of large and presumably ancient impact basins are preserved as inliers within the lowland plains.

Sleep sets out several ways by which his ideas could be tested. The duration of genesis of lowlands crust in his reconstruction is 200–300 million years, possibly sufficient to discern an age gradation from variations in impact crater densities. The subsidence of thermally generated topography may give rise to differential vertical motions along older channels and lava flows. By the same process, the present lowlands will have subsided relative to the southern uplands, perhaps reversing the sense of escarpment along the passive margin at a time significantly later than the initial breakup. The patterns of gravity anomalies across passive margins, extinct subduction zones and fossil fracture zones may be distinguishable and detailed mapping of the geology of key features may resolve their origin. Finally, the hydrothermal alteration of the upper crust and the subduction of water might have been responsible for removing surface water, so studies of the

role of water in magma generation beneath the Tharsis Montes, and of how the history of water on Mars dovetails with the proposed history of plate subduction, offer promise as further tests.

Sleep's proposal is a shot across the bows of the conventional view of martian geological history, and vigorous debate can be expected. But the notion that we may after all have a record of plate tectonics on another planet and that it may, unlike that of the Earth, include a record of global mantle cooling is sufficiently provocative that such a debate is welcome. □

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STAR FORMATION

Hubble looks at a stellar nursery



STAR formation occurs in clouds of gas — composed primarily of molecular hydrogen (H_2) — when portions of the cloud collapse under the influence of gravity. It is generally accepted that the later stages of this collapse are associated with a disk of gas and dust surrounding the young star, and evidence for such disks around about half of the young stars in the Orion Nebula (the middle 'star' in Orion's sword) has now been seen by C. Robert O'Dell and Zheng Wen, using the newly refurbished Hubble Space Telescope — of the five young stars shown above, four have disks. Planets are thought to coalesce in the disks as a natural part of the star-formation process. A nice cartoon of this can be seen in the opening credits of the television show *Star Trek: The Next Generation*.

The work by O'Dell and Wen follows years of study of related disks in the Taurus and Ophiuchus molecular clouds, using infrared and millimetre-wavelength observations, by Beckwith, Strom and Andre and collaborators, who first found evidence for disks surrounding about half of all young stars. The new observations are important because we can see directly the outer boundaries of the disks, where the gas is being heated (by radiation from a nearby

massive star) to the extent that it is ionized. As the electrons recombine with the atoms, distinctive light is emitted, allowing astronomers to estimate the mass of hot gas, which is probably much less than the total mass of the disk. O'Dell and Wen find that there are typically a few Earth masses of ionized gas associated with their disks. (The masses traced by the infrared and millimetre-wavelength observations are usually much greater.)

If disks are relatively common around young stars in our Galaxy, planets like those in our Solar System might also be common. As described on pages 610 and 628 of this issue, a near-infrared image of the disk around the main-sequence star β Pictoris shows evidence for the presence of planets. β Pic is being seen at a later stage of evolution than the stars studied by O'Dell and Wen — much of the gas and dust associated with its birth have been dissipated — but the results are complementary and suggestive. We see first the disks around young stars, and later the traces of planets as the disks disappear. The final step — seeing the planets themselves — still lies in the future.

Leslie Sage

Pitcairn before the *Bounty*

Jared M. Diamond

DESPERATE to escape detection by a vengeful British Admiralty, in 1790 the mutineers of HMS *Bounty* chose Pitcairn Island, then uninhabited, as their hiding place because of its remoteness. For Pitcairn and its neighbours, Henderson, Ducie and Oeno, the nearest lands of any sort are some other small Polynesian islands 450–1,300 km distant, while the nearest continents (South America and Australia) are more than 4,500 km across the Pacific. That same remoteness is what attracts biologists to the Pitcairn group. Because it is so difficult for plants and animals (as well as scientists) to get to the islands, they are home to few species, but a large proportion of them are endemics organized in very simple ecosystems. This then, is a scientifically remarkable part of the world, and a meeting last month* was devoted to discussion of the results of an expedition which investigated the islands' natural history.

The Pitcairn group's biota is a mixture of species also found on other tropical Pacific islands, and of endemic forms that evolved on the group itself (T. Benton, Univ. East Anglia; R. Irving, Pulborough; R. Preece, Univ. Cambridge; S. Waldren, Trinity College Botanic Garden, Dublin; G. Wragg, Univ. Oxford). There are few or no endemics among species groups that disperse readily over water — such as spiders and marine molluscs and fish — because of continuing high gene flow from populations elsewhere. Rather, the endemics have formed among groups, such as land snails, land birds, weevils and oribatid mites, that have colonized the islands only with difficulty. The geological ages of 450,000–900,000 years for Pitcairn and 285,000 years for Henderson place upper limits on the time available for these species to have evolved (T. Spencer, Univ. Cambridge; S. Blake, ANU, Canberra). For Henderson, that biological history is stunningly displayed in the fossil coral lagoon that now, uplifted 22 m above sea level, constitutes the island's interior. Dozens of species of fossil corals, giant clams and other fossil molluscs are still well preserved and identifiable on that dry aerial reef (S. Blake; R. Preece).

Some of Henderson's endemic species have evolved distinctive adaptations to the island's environment. For example, most relatives of the Henderson fruit dove are flock-forming birds that live in wet

forests on big islands with high diversity of fruiting trees. Henderson, however, has no permanent fresh water and only a dozen tree species with edible fruits, of which only a few species may be bearing fruit in any given month. Faced with these difficulties, the Henderson fruit dove has evolved the habit — possibly unique among birds — of eating fern shoots with a 94 per cent water content instead of

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Pitcairn Island — splendid isolation.

drinking (P. Jones, Univ. Edinburgh). Exceptionally among fruit doves, it is also apparently solitary and territorial: a dove cruises its territory each day, knows the location of all relevant fruit trees, tests their fruit daily for ripeness, and finally eats each fruit on the first day that it ripens.

How did the ancestors of the Pitcairn islands' biota arrive across hundreds or even thousands of kilometres of ocean? The group illustrates well the various solutions to this fundamental problem of island biology. Some species, such as birds, arrived by flying there (M. Brooke; P. Jones; G. Wragg; R. Trevelyan, Univ. Oxford). Others arrived by being blown passively: many of Henderson's terrestrial invertebrates belong to groups regularly caught as aerial plankton in nets towed behind aircraft (T. Benton). Woodlice and mites arrived on rafts of floating vegetation. Marine species arrived as oceanic plankton — but with difficulty, because the surrounding waters are too cool in most years for coral larvae to survive (R. Irving; T. Spencer). Larvae instead colonize mainly in the occasional El Niño year, when a tongue of warm water spreads eastwards across the Pacific. Finally, some species were introduced intentionally (coconuts) or unintentionally (rats) by the Polynesians who preceded the *Bounty* mutineers (S. Waldren; M. Weisler, Historic Preservation Office, Majuro, Marshall Islands), by the

mutineers themselves, and by modern visitors.

If you looked at a map of the Pacific and tried to guess the direction (South America in the east or New Guinea in the west) from which most species had arrived at the Pitcairn group, you would guess South America because it is closer. Knowledge of prevailing winds and ocean currents (from the east) would reinforce you in your opinion. Yet you would be wrong: almost all of the islands' plants and animals came from the west (T. Benton; R. Preece; S. Waldren; G. Wragg). Only a woodlouse, a fly, a couple of centipedes and a few other species came from the east. The same paradox characterizes other Polynesian islands: even the Polynesian people themselves arrived upstream from the west (P. Kirch, Univ. California Berkeley), contrary to Thor Heyerdahl's initial view.

As a more careful examination of a map will suggest, the explanation is that the western but not the eastern tropical Pacific is dotted with island targets that selected for the evolution of over-water colonization ability. Polynesian plants and animals, including Polynesian people, arrived especially during the occasional periods of reversed westerly winds and currents. Thus, the evidence from Pitcairn adds weight to the principle that dispersal ability evolves only when the potential payoff of occasionally founding a new population selects for it.

Conversely, the islands' biota also illustrates the paradox that has most puzzled and misled biogeographers: island occurrences of taxa that are flightless and lack other means of crossing water. For instance, the Pitcairn group has flightless endemic birds (P. Jones; G. Wragg) and weevils (T. Benton), and endemic marine molluscs lacking planktonic larvae (R. Preece). Biogeographers used to attribute the arrivals of such taxa to supposed land bridges, now vanished. Had all such postulated bridges existed, the oceans would have been virtually paved over.

Instead, these stranded, now-sedentary endemics on the Pitcairns arose from mobile ancestors whose means of dispersal were subsequently eliminated by natural selection after they colonized the islands. For example, the flightless Henderson rail evolved from the wide-ranging spotless crane, while the molluscs lacking planktonic larvae evolved from ancestors possessing them. This illustrates the point that dispersal mechanisms not only bring potential benefits but also incur costs. Planktonic larvae, birds' flight muscles and offspring wafted into the aether are expensive to produce. They were quickly selected out of populations that colonized a stable, isolated environment, as was the

Michael Brooke

*The Biogeography, Ecology and Prehistory of the Pitcairn Islands, Linnean Society, London, 5–6 May 1994.

case with the dodo of Mauritius and so many other island endemics.

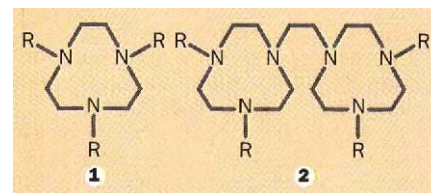
Beyond these lessons, the results of the Pitcairn Expedition convey a much broader message. The golden days of biological exploration are now usually regarded as long past. It is widely assumed that most major biological discoveries awaiting us will be made in laboratories, and that the few remaining biological explorers are romantics seeking an excuse to satisfy their wanderlust. But most of the world's plant and animal species remain undescribed, the habits of most described species remain unknown, and investigations of those habits often yield remarkable discoveries. *Escherichia coli* will forever

be available for us to study, but sadly the Pitcairn islands' endemic species will not. Despite its remoteness, most of Pitcairn's summit is now overrun with the introduced rose-apple tree (*I. Hepburn*, Fen Ditton), the shores of its atolls are littered with garbage washed across the oceans, and its breeding seabirds are being decimated by rats. The Pitcairn Expedition may prove to have been one of our last chances to study one of the world's most isolated biotas. □

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facturers have had to do is to comb this literature, seeking systems or compounds that act as selective organic oxidants and also satisfy a plethora of practical requirements — performance, toxicology, environmental acceptability and cost. Unilever now claims to have found such a system. The chemistry underlying the performance is described in this issue¹; the toxicological and environmental aspects will doubtless be discussed elsewhere.

The key to the invention is the discovery⁵ that complexes of manganese with derivatives of the nitrogen heterocycle triazacyclononane (TACN, **1** in the figure) and its dimer (**2**) are efficient, selective oxidation catalysts at ambient temperatures. Manganese porphyrins, whose molecules contain four nitrogen atoms linked to the manganese atom in conjugated systems, function as oxidases in biological systems; the TACN systems discussed here differ from them in having only three nitrogen atoms linked to the manganese in unconjugated systems. The hydrogen peroxide is provided by one of the solid compounds mentioned above. The active oxidizing species has yet to be identified, and different species may operate at different pH values. Mixed-valence compounds, containing two manganese atoms in different valence states, may be involved.



Hage and colleagues do not reveal the ultimate environmental fate of the manganese in their paper, but a press release says that "it is neither deposited on the fabrics nor discharged into the environment in a form which could be damaging". Presumably it becomes lodged in the zeolite from the detergent by ion exchange and finishes up in sewage sludge.

The epoxidation experiments that Hage *et al.* describe were done in order to learn more about the bleaching process, and they show that epoxidation and bleaching probably proceed by different routes. Organic chemists will want to find out whether this type of catalysed epoxidation has synthetic uses too. Heterogeneous oxidation systems, using hydrogen peroxide and catalysed by titanium incorporated in a siliceous zeolite, are used indus-

DETERGENTS

Bleaching catalyst revealed

Alan E. Comyns

ON page 637 of this issue, Ronald Hage and colleagues at the Unilever laboratories in The Netherlands and New Jersey describe a new family of homogeneous oxidation catalysts for use as low-temperature bleaches in detergents¹. The announcement coincides with the launch by Lever Brothers of two new detergents (Persil Power in the UK and Omo Power elsewhere in Europe) containing one of the catalysts ('AcceleratorTM'). The launch stirred up an almighty row between the rival detergent manufacturers Lever and Procter & Gamble, which was described in last week's issue².

'Bleaching' in this context involves oxidizing stains. The fundamental problem in bleaching textiles, whether in the manufacture of the cloth or in its subsequent laundering, is how to oxidize the colour without oxidizing and thereby weakening the cloth. Both the colour and the cloth are organic materials, and a powerful oxidant will attack both. In an industrial bleaching process during textile manufacture, a variety of oxidants (including halogens and their oxyacids), and even reductants, can be used. But on the domestic scene, for incorporation into a detergent formulation, the only currently acceptable oxidants are those based on hydrogen peroxide. For powdered detergents, this hydrogen peroxide is provided in the solid form by the use of a sodium perborate or sodium carbonate perox-

ohydrate, both of which liberate hydrogen peroxide when dissolved in water.

Hydrogen peroxide alone is an effective bleaching agent above about 60 °C. Many of today's fabrics must be washed at lower temperatures; for this reason, as well as to save energy, the hydrogen peroxide must be converted into a more powerful oxidant. For many years the detergent manufacturers have been seeking the ideal low-temperature oxidant. The usual approach has been to use compounds that convert hydrogen peroxide to peroxy-carboxylic acids when they are dissolved in water. Oxidations with such reagents are stoichiometric processes, not catalytic ones, so compounds must be used in relatively high concentrations and therefore have to be cheap. A catalytic process would have obvious advantages, provided that the fundamental problem stated above can be solved.

The oxidative power of hydrogen peroxide is greatly enhanced by transition metal ions: so much has been known since the last century, when H. J. H. Fenton invented his analytical reagent ($\text{Fe}^{2+} + \text{H}_2\text{O}_2$), and textile bleachers recognized the tendency of rust particles to make pinholes in cloth. The active species here is the hydroxyl radical, generated by $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\bullet$. The hydroxyl radical is such a strong oxidant that Fenton's reagent is now used commercially to destroy organic pollutants in industrial effluents^{3,4}. But in general it is too strong and too unselective to be used in either organic synthesis or textile bleaching.

Interactions between hydrogen peroxide, transition metal ions, and organic compounds started to be studied soon after hydrogen peroxide was discovered in 1818, and the literature on them is now very extensive. What the detergent manu-

Erratum

In J. Boissonade's News and Views article of 19 May ('Chemical patterns: Long-range inhibition') the name of J. E. Pearson of Los Alamos National Laboratory was inadvertently omitted from ref. 3, the principal paper under discussion. That reference should read Lee, K.-J., McCormick, W. D., Pearson, J. E. & Swinney, H. L. *Nature* **369**, 215–218 (1994).

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2. Verrall, M. *Nature* **369**, 511 (1994).
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RÉSUMÉ

Well-timed words

WOULD-BE linguists should pay as much attention to the rhythms and timing of words in foreign tongues as to pronunciation of individual sounds. That was the conclusion of K. Tajima and R. Port (Indiana Univ.) and J. Dalby (Communication Disorders Technology, Indiana) at a meeting of the American Acoustical Society earlier this month. They recorded the same short English phrases spoken by a native English speaker and by a native Chinese speaker, digitized the waveforms, and did a little fancy cutting and pasting to expand or contract parts of the Chinese speaker's phrase so that the timing matched that of the English speaker. Listeners presented with the unadulterated phrase and asked to say which of two similar-sounding options they had heard (say, "equal size" or "you're concise") identified the correct meaning in only 55 per cent of cases, whereas for the re-timed phrases the success rate rose to about 80 per cent.

Pack deaths

SMALL populations of endangered species may be driven to extinction by the very researchers whose concern it is to keep the animals alive. This dark possibility emerges from further evidence that the packs of wild dogs (*Lycaon pictus*) in the Serengeti and Masai Mara that became extinct between 1965 and 1991 were the same ones studied by researchers. R. Burrows *et al.* (*Proc. R. Soc. Lond. B* **256**, 281–292; 1994) re-evaluate their own and others' data, and alternative hypotheses, and conclude that the most likely explanation for the demise of the study packs is that intervention-induced stress made the animals more vulnerable to rabies. The message is that the effects of field techniques such as trapping, darting, tagging, radio-collaring, tissue sampling and vaccination should not be ignored as factors influencing the fate of species under threat.

Orbital link

HYDROGEN bonds between oxygen and hydrogen are common throughout organic chemistry; fluorine and nitrogen, two other highly electronegative atoms, also form such bonds readily. But M. Iwaoka and S. Tomoda (*J. Am. chem. Soc.* **116**, 4463–4464; 1994) believe they have now found a compound held in shape by hydrogen bonds to selenium. NMR and single-crystal X-ray data on diselenocin, which contains two benzyl rings linked by a larger, floppier ring of two selenium and two CH₂ units, lead them to conclude that each selenium attracts one of the opposing hydrogen atoms. This is unexpected, as selenium is not highly electronegative, nor is the C–H bond highly polar. The authors suggest that it arises from orbital interaction between the selenium lone pair and C–H.

trially by Enichem in Italy to oxidize phenol to catechol and hydroquinone, and to make cyclohexanone oxime from cyclohexanone and ammonia⁶. Many homogeneous oxidation systems, using hydrogen peroxide and catalysed by transition metal ions and complexes, are

known in the laboratory, but as yet there are no published examples of commercial applications. □

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PLANETARY SCIENCE

Footprints in the dust

Charles M. Telesco

NEW infrared images of a disk of particles circling the star β Pictoris are the testing ground for a powerful indirect means of detecting other planetary systems, as Lagage and Pantin show on page 628 of this issue¹. The images reveal that the inner region of the disk is clear of dust, possibly swept away by a planet.

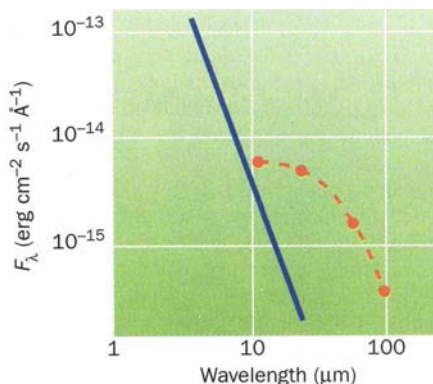
The only direct way to detect an extra-solar planet is to image the star and look

lines from the star's photosphere. Studies of stellar line-of-sight motions provide strong evidence for the existence of several planetary systems beyond our own^{2–5}. The most spectacular of these is the pulsar B1257+12, for which the periodicity of the radio pulses from the neutron star seems to be modified by at least two planets with masses about three times that of the Earth, tugging on the star^{5,6}.

But a planet's gravitational pull can have subtler influences in a stellar system. Besides the Sun, planets and comets, our Solar System contains solid particles with sizes ranging from many kilometres (asteroids) to nanometres (dust). Our Solar System's zodiacal light is actually sunlight scattered from dust particles distributed throughout the orbital plane of the planets. The particles reflect only part of the incident sunlight, absorbing the rest which is then emitted at infrared wavelengths. The Infrared Astronomical Satellite (IRAS) mission in 1983 showed that the gravitational influence of the planets has caused the zodiacal dust to be distributed in distinct bands⁷. Can we detect the visible or infrared radiation from such particles in other star systems? If so, perhaps a planet there would leave its gravitational footprints in the dust just as it has in our Solar System. The infrared images of β Pic made by Lagage and Pantin indicate that this elegant indirect approach to detecting planets may indeed be feasible.

Using IRAS, astronomers were amazed to discover that the main-sequence star Vega has ten times more emission at wavelengths of 60 and 100 μ m than expected from a reasonable extrapolation of the visual flux⁸. More than a hundred such stars have now been found, β Pic being the most remarkable example. Visual images of β Pic show what is surely an edge-on disk extending out to more than 1,000 astronomical units from the star⁹ (1 AU is the distance from Earth to Sun). As in the Solar System's zodiacal disk, the extended emission around stars in this class results from both scattering and absorption of starlight by disk particles.

The disk of β Pic appears unusually bright and large (its angular size is more than an arcminute), and it is thus a special



The infrared excess emission (points) detected from the star β Pictoris by IRAS, compared with the normal emission from the star (solid line). Near 10 μ m, the total emission from the inner disk, imaged by Lagage and Pantin¹, is about equal to that from the star itself. The star's visible light peaks near 0.5 μ m at a flux (F_λ) of $\log F_\lambda \approx -10$.

for the spot of planetary light nearby. The task is extraordinarily difficult because the planets can be lost in the glare of the much brighter star. The reflected solar radiation from Jupiter, the largest planet in our Solar System, is a billion times fainter than the Sun at visual wavelengths, and viewed from a modest distance of 5 parsecs (16 light years), Jupiter would be separated by less than an arcsec from the Sun. This approach, so far unsuccessful, will benefit greatly from the advent of new technologies and bigger telescopes².

Indirect search techniques take advantage of the fact that planets gravitationally disturb their environments. For instance, a planet and its star orbit their centre of gravity, causing a periodic wobble of the star's position on the sky and along the line of sight, the latter motion detectable by the Doppler shift of spectral absorption

target for detailed studies. To understand planetary evolution, many astronomers are focusing on the region within 30 AU of the star, as the planets in our Solar System occupy a region of that size. But at optical wavelengths the star is orders of magnitude brighter than the inner disk region, and the spillover of the light from the star swamps the starlight scattered by the inner disk in images made with optical charge-coupled devices^{9,10}. In the infrared, the inner disk is much brighter relative to the star (see figure), but until recently the angular resolution of the most sensitive infrared cameras was too poor to reveal the details of the inner disk.

Lagage and Pantin's image at a wavelength of 10 μm is the first high-resolution infrared image of the emission from particles in a circumstellar disk and the first direct detection of a central hole in the disk. Such an inner region depleted of dust particles had been inferred previously from lower-resolution infrared observations: the expected emission from the hottest particles (those that would be orbiting nearest the star) was absent^{11,12}. The newest images confirm that conclusion, but, better still, they establish the size and sharpness of the depletion zone: it extends to about 15 AU from the star and has an average particle density only a tenth of that just outside the zone.

Because it is about the size of our Solar System, Lagage and Pantin speculate that the inner zone may be swept clean by the gravitational pull of a planet orbiting there. In this picture, which is supported by theoretical calculations¹³, a planet gravitationally deflects the particles out of the inner zone or into highly eccentric orbits that take them much closer to the star where the increased efficiency of radiation drag (the Poynting–Robertson effect) quickly leads to their infall onto the star. As particles from the outer disk spiral inwards in response to various radiation and gas forces, and as new particles are released by comets and asteroid collisions, the planet shepherds them away, keeping the zone relatively clean. This hypothetical planet may also be deflecting comets onto the star, as indicated by the presence

of highly variable absorption lines in the spectrum of β Pic¹⁴.

The infrared image by Lagage and Pantin provides a special bonus: the edge-on disk is not symmetric about the star. An asymmetry is also seen in visual images of the outer disk region, but the new images suggest a more intimate relationship between the asymmetry and the properties of the inner disk. As the orbital timescale for particles in the inner disk is relatively short (less than 100 years), one expects disk irregularities to be smoothed out fairly quickly unless something is stirring

them up. That something could be a planet¹⁵.

Although pleasing, this explanation is far from ironclad; the disk in β Pic is an everchanging entity experiencing transient instabilities and features which are related to its complex history. Nonetheless the new infrared images demonstrate a powerful new probe for possible planetary systems. □

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G PROTEINS

The importance of being GTP

Henry R. Bourne

ON page 621 of this issue¹, Lambright *et al.* describe the crystal structure of the α -subunit (G_{α} or α_1) of a trimeric G protein, retinal transducin, in its GDP-bound form. Seven months ago, investigators in the same laboratories reported² the structure of G_{α} bound to GTP- γ S.

Although the α -carbon backbones of the two structures are virtually identical for 86% of the positions examined in both,

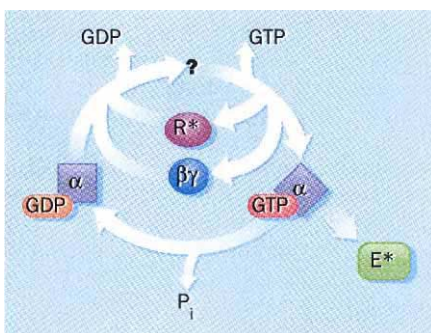


FIG. 1 The GTPase cycle of a trimeric G protein. After the GDP-bound conformation of G_{α} (square) binds to $G_{\beta\gamma}$, an activated receptor (R^*) triggers dissociation of GDP. GTP then binds, and receptor and $G_{\beta\gamma}$ dissociate from the GTP-bound conformation of G_{α} (diamond), which can then activate the appropriate effector (E^*). Hydrolysis of bound GTP converts α -GTP back to α -GDP, and the cycle repeats.

the present report merits comment in News and Views, as did the first³. Aside from a devoted coterie of G-protein watchers, why should biologists care about the 14% difference?

The answer is that the γ -phosphate of GTP (which distinguishes it from GDP) triggers the conformational changes in G_{α} that allow it to transmit visual signals in flies, frogs and humans⁴. (Because it resists hydrolysis, the γ -thiophosphate of GTP- γ S was used² as a stand-in for the γ -phosphate.) At least 16 mammalian G proteins, built on the same architectural plan as retinal transducin^{5–7}, use the γ -

phosphate of GTP to transduce signals from receptors for extracellular stimuli into altered activities of the effector proteins (enzymes and ion channels) that mediate cellular responses to photons, odours, hormones and neurotransmitters.

Together, the two G_{α} structures furnish contrasting freeze-frame pictures of two key intermediates in the G-protein cycle (Fig. 1); we can now see what difference a γ -phosphate makes, by comparing the inactive conformation, α -GDP, with its active counterpart, α -GTP (Fig. 2). Interacting with only three amino acids^{1,2}, the γ -phosphate lifts switch I (red) upwards and twists switch II (orange and cyan) so that it contacts and pulls switch III (yellow) slightly towards the right. These concerted movements bunch the three switch regions of α_1 -GTP into a protuberant hump that diagonally crosses the face of the α_1 -GTP structure shown in Fig. 2.

How does this diagonal hump make the GTP-bound form of G_{α} behave so differently from the GDP-bound form? α -GTP dissociates rapidly from G-protein $\beta\gamma$ ($G_{\beta\gamma}$) subunits, but binds to and stimulates effectors, while α -GDP behaves in exactly the opposite fashion — it binds tightly to $G_{\beta\gamma}$ but not to effectors, and (in combination with $G_{\beta\gamma}$) is capable of interacting efficiently with receptors (Fig. 1). These key differences in interactions with three other proteins all result from changes in a small surface area located on one face of G_{α} ; switch regions I–III contain only 14% of the amino acids examined in α_1 -GDP (ref. 1).

In unstimulated cells, $G_{\beta\gamma}$ is sequestered by binding to α -GDP; its dissociation allows free $G_{\beta\gamma}$ to activate a number of effectors, including specific phospholipases and K^+ channels. $G_{\beta\gamma}$ interacts with a surface of α -GDP that includes residues in or close to switch II, as suggested by a point mutation in α_s that blocks dissociation of α_s from $G_{\beta\gamma}$

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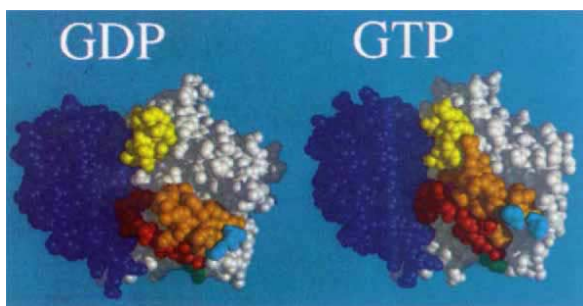


FIG. 2 Space-filling models showing the switch regions (red, orange, cyan, yellow) of α_1 -GDP and α_1 -GTP, as described in the text. Bound guanine nucleotide (not visible) is located in a binding pocket between the Ras-like core domain (white) and the α -helical domain² (dark blue) that stimulates its GTPase activity¹⁶. (Coordinates of the two structures^{1,2} provided by Paul Sigler.)

(refs 8,9), apparently by preventing GTP-induced conformational change in the $\alpha 2$ helix of switch II. Moreover, the β -chain of a G_o heterotrimer can be crosslinked to a cysteine in the switch II region of α_o (ref. 10) and genetic analysis in *Saccharomyces cerevisiae* suggests that the α - $\beta\gamma$ interaction involves a G_α residue that is cognate to the green residue (Q184 of α_i) in Fig. 2, near both switch I and switch II. As an additional potential $G_{\beta\gamma}$ interaction site, Lambright *et al.* point to the shallow groove in α_i -GDP, just above switch II, which is filled in by part of the diagonal hump (mostly switch II) in α_i -GTP.

A second consequence of the conformation induced by the γ -phosphate is that it converts G_α into a protein that can activate effectors directly; examples include activation of adenylyl cyclase by α_s and of retinal cyclic GMP phosphodiesterase by G_{ta} . Although effectors must interact, at least in part, with conformationally changed surfaces of G_α , site-directed α_i mutations^{11,12} and biochemical effects of G_{ta} peptides¹³ identify effector-interacting residues in these two G_α proteins which are located for the most part in surface regions unaffected by conformational change; these regions (not coloured) are located near the top right edge of the side of G_α depicted in Fig. 2. The switch regions do contain at least one putative effector-contact region, however; in α_s , this short stretch of sequence^{11,12} corresponds to residues (cyan in Fig. 2) in the turn that follows the $\alpha 2$ helix of switch II in G_{ta} .

The proximity of identified effector-interacting residues (cyan) to residues that interact with $G_{\beta\gamma}$ (switch II and the green residue in Fig. 2) makes it likely that the $G_{\beta\gamma}$ - and effector-interacting surfaces of G_α overlap significantly within the small surface area occupied by the switch regions. The overlap means that G_α cannot interact with an effector until it divests itself of the $\beta\gamma$ subunits.

The two G_{ta} structures do not, however, shed much light on a third protein-

protein association, one essential for signal transmission but dependent on the absence of the γ -phosphate — that is, binding of both G_α and $G_{\beta\gamma}$ to the receptor (Fig. 1). Physiologically, γ -phosphate-induced reversal of this association is important because it allows a single receptor molecule to activate additional G-protein molecules, thus amplifying the extracellular signal. The reversal may be explained, at least in part, as a consequence of GTP-induced dissociation of the α from the $\beta\gamma$ subunits, in that $G_{\beta\gamma}$ appears to be required for

effective interaction of the receptor with G_α (ref. 14; Fig. 1). Nonetheless, it is striking that most of the G_α surfaces thought^{2,15} to interact with receptors are on the side of the protein opposite the surface (Fig. 2) that changes conformation in a GTP-dependent manner. A possible exception is the protein's extreme carboxy terminus, which was found (in one of three α_i -GTP- γS protomers²) juxtaposed to the turn containing the effector-interacting (cyan) residues in Fig. 2.

The receptor-G-protein puzzle sets a third and yet more formidable challenge for doughty G-protein crystallographers — show us a freeze-frame picture of the 'empty' state of G_α (the question-mark in Fig. 1), the mysterious conformation, stabilized by activated receptor and $G_{\beta\gamma}$, that serves as a transition state in the GTP/GDP exchange reaction. Now that we know what a difference a γ -phosphate makes, we want to know how it gets there. □

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DAEDALUS

The long view

THE human eye cannot hold an image for long. Film and television, which repeatedly present a new image before the old one has faded, give the illusion of continuous motion. Daedalus argues that this persistence of vision is not merely a form of retinal inertia. If it were, a bright image should persist for longer, as it does in a television camera. In fact, the eye's image-fusion rate is about 50 Hz in bright light, but only 10 Hz in dim light — hence the advantage of a dimly-lit cinema. Daedalus reckons that the rate is set by the visual pathways. He points out the curious fact that the human image-fusion frequency falls with rising hydrostatic pressure.

At first he began to dream up an underwater cinema for deep-sea divers, in which even jerky old films with their low frame-rate would appear smooth and continuous. But he then recalled that pressure affects many biochemical processes by altering the permeability of cell membranes. Thus it can slow the swimming of *Paramecium* by blocking the entry of calcium ions into its cilia.

Many drugs can also work this trick. They can bind to a specific ion and alter its diffusion rate far more dramatically than any feasible hydrostatic pressure. Daedalus suspects that drugs such as mescaline and LSD act like this. By disrupting the time constants of the user's visual pathways, they distort his visual impressions and sabotage his time sense. So DREADCO's biochemists are now modifying these drugs to fine-tune this action. Their goal is a drug that has no psychedelic effects, but greatly extends the persistence of vision.

DREADCO's 'Slowview' may merely induce a drugged nightmare of lagging and persistent images. But Daedalus hopes that, by slowing the whole time-dependent process of visual interpretation, it will in effect convert the eye into a time-lapse camera. A new world of leisurely movement will spring to life. The user of Slowview will retain his normal internal clock and reaction time, but his effective 'visual present' will extend to many seconds. He will appreciate optical changes too gradual for the rest of us to notice: the slow convolutions of a cloudscape, the opening of flowers, the changing shadow patterns of a forest, the growth of crystals and even that cliché of ultimate boredom, the drying of paint. With the aid of Slowview, a lantern-slide lecture will become a true movie, botany will become a fast-moving subject and sluggish reptiles such as skinks will appear as frisky and vivacious to us as they doubtless do to each other.

David Jones

How to observe comet impact

SIR — While most of the attention surrounding the July 1994 impact of comet Shoemaker–Levy 9 on the far side of Jupiter has focused on the ~ 20 'large' (diameter $D \approx 1$ km) fragments, there might also be many 'small' ($D \approx 100$ m) fragments in the meteor stream that have gone undetected (ref. 1, for example). The explosion of these small fragments in the jovian atmosphere may be luminous enough briefly to brighten Jupiter's nearest galilean moon, Io, when in eclipse to a level observable through relatively small telescopes on Earth. The reflection events will contain useful information regarding the total number, size distribution, density and material strength of small fragments from the tidally disrupted comet. Serious amateur astronomers can play a useful role in obtaining such information.

Simple scaling of the calculations of Hills and Goda² for Earth meteors to the impact of Shoemaker–Levy 9 on Jupiter suggest that a $D = 200$ m comet with a density of 0.5 g cm^{-3} that enters the jovian atmosphere at 60 km s^{-1} and a zenith angle of 45° should explode well above Jupiter's clouds, and would have a peak apparent visual magnitude of $V \approx -1.2$, if it could be directly observed from Earth. For comparison, Jupiter will have $V = -2.1$. Because the comet's impacts are expected to occur on the far side of Jupiter, the meteors are unlikely to be directly observable from Earth. However, Io in eclipse (normally invisible) would brighten to an apparent visual magnitude of $V \approx 11$ as seen from Earth due to the reflection of the explosion of the $D = 200$ m impactor described above. The reflection off Io should be within one magnitude of the peak value for roughly half a second.

Because there may be small fragments spread throughout the comet's debris train, all six eclipses of Io from 15 to 24 July are worth observing. The table gives the times of the ends of these eclipses, and the best place from which to observe them. The latter are all in the Southern Hemisphere, as Jupiter will be south of the celestial equator and in the western sky after evening twilight. Note that large cometary fragments will strike Jupiter an hour or so after the 18 and 22 July eclipses (P. W. Chodas and D. K. Yeomans, personal communication). Consequently, small meteors may be more frequent during these two eclipses.

Faint reflections off Io will be difficult to see because of the glare from Jupiter. There will be ≥ 1 h before the end of an eclipse during which Io is not occulted by Jupiter. When Io reappears, it will be ≥ 1 jovian angular radius away from the dark edge of Jupiter. A ≥ 14 -inch telescope will be required for an average observer to see

a $V \approx 11$ flash at this angular distance from Jupiter³. An even larger telescope will be required to detect such a flash earlier in an eclipse when Io is closer to Jupiter's limb. The final half hour of an eclipse will provide the most realistic observing opportunity for amateurs.

Visual observers should use the highest practical magnification, and have an accurate clock available. It is important for an observer to record his or her location

Times of eclipse reappearances of Io from 15 to 22 July 1994

Geocentric UT of reappearance*	Observable from†
July 15, 5 h 40 min	South Pacific Ocean
July 17, 0 h 09 min	South America
July 18, 18 h 38 min	Eastern half of Africa
July 20, 13 h 07 min	Southeast Asia, Australia
July 22, 7 h 36 min	South Pacific Ocean
July 24, 2 h 04 min	South America

*From the 1994 *Observer's Handbook of the Royal Astronomical Society of Canada*.

†Based on plots by L. H. Wasserman of Lowell Observatory.

(longitude and latitude), the aperture and type of telescope used, and the magnification. A tape recorder will allow information to be recorded without interfering with observation. The time should be noted on the tape before and after each continuous observing session. For each obvious and possible Io flash observed, one should record an estimate of the peak brightness, the duration of the event, and how convinced the observer is that the flash was real. The exact time of each flash can be recovered afterwards by listening to the tape and using a stop watch. Also important are the time interval over which the observations are made, how complete the coverage is during this interval, and an assessment of how good the observing conditions are. The observing session may end once Io has fully reappeared from eclipse. Note that the lack of a detection through a ≥ 14 -inch telescope in good observing conditions is still a scientifically useful result. All observations sent to me at the address below will be made available to professional astronomers involved in the study of the Shoemaker–Levy 9 impact event.

There is an eclipse of Europa on 19 July from 9 h 42 min to 12 h 04 min UT during which a large fragment of Shoemaker–Levy 9 is expected to hit Jupiter. It is possible that Europa will brighten from complete invisibility to a level observable by amateur astronomers using small telescopes for tens of seconds. This event will

be visible from Australia and New Zealand. Note that it would be useful to observe the entire eclipse using large telescopes to search for faint flashes. Also, as Io is larger and closer to Jupiter than Europa, flashes from small meteors will be brighter off Io in eclipse than off Europa in eclipse. Therefore, the six eclipses of Io from 15 to 24 July are still worth observing.

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Mercury in Brazil

SIR — Nriagu *et al.* report¹ severe environmental mercury contamination in the Madeira river basin in the Brazilian Amazon. Their results, together with previous studies, suggest that the use of mercury by informal sector miners in Amazonia may pose a considerable health risk both for the miners themselves and for the populations of mining areas^{2,3}. We present the results for blood and urine analysis on 106 individuals from four sites in the Tapajós river valley, northern Brazil. These show that mercury levels are high enough to produce clinical symptoms of mercury poisoning. A particular cause for concern is the high level of blood mercury in villagers eating fish caught locally.

Production in this area has been estimated at 12–20 tonnes of gold annually since 1979, with a similar discharge of mercury into the regional ecosystem. The city of Itaituba (population about 100,000) is the most important gold-trading site in Amazonia. The other locations sampled were the gold-mining camps of Crepurí (population about 3,000) and Cuiú-Cuiú (population about 1,000), and the fishing village of Jacareacanga on the river Tapajós, 100 km from the nearest mining camps. Blood and urine samples were collected by a physician at improvised clinics, frozen within 48 hours, and Hg was determined using inductively coupled plasma source mass spectrometry (ICP-MS) with stringent quality-control procedures.

The distinct risk categories defined were: miner; trader (gold traders for whom burning mercury/gold amalgam was part of work routines); resident (person living in or near building where burning regularly occurred but not directly engaged in mining or trading); child (resident aged below 16 years); pregnant women; and fish-eaters (Jacareacanga only; low-income residents with fish as basis of diet) (see table).

The two main pathways of human expo-

MERCURY CONCENTRATION IN BLOOD AND URINE BY RISK GROUP (ARITHMETIC MEAN)

	No. in sample	Hg in blood ($\mu\text{g l}^{-1}$)	Hg in urine ($\mu\text{g l}^{-1}$)
Trader	42	30.4	78.9
Miner	18	34.3	18.5
Pregnant female	8	14.1	10.0
Fisheater: adult	14	96.7	13.9
Fisheater: child	5	70.4	6.4
Resident: adult	4	20.0	18.5
Resident: child	16	14.5	18.6

sure are the inhalation of inorganic Hg vapour when mercury/gold amalgam is burnt, both at the site of extraction and when it is subsequently traded, and the ingestion of methylated (organic) Hg in fish tissue. Mercury in urine is an appropriate indicator of the former. At urinary Hg concentrations of $100 \mu\text{g creatinine}^{-1}$ (approximately $100 \mu\text{g l}^{-1}$) there is a high probability of developing the classic signs of mercurialism, erethism and tremor. At Crepuri, six individuals exceeded $100 \mu\text{g Hg l}^{-1}$, all of them traders, three of whom showed levels above $200 \mu\text{g l}^{-1}$ Hg, the highest level recorded being $843 \mu\text{g l}^{-1}$ Hg. In all, 22 of the 106 individuals sampled exceeded or approached $50 \mu\text{g l}^{-1}$ Hg, a level at which industrial workers have been recommended to be removed from further exposure⁴.

Mercury in blood is an appropriate indicator of recent exposure to ingestion of organic Hg in fish. In a population with high fish consumption, a blood Hg level of the order of $200 \mu\text{g l}^{-1}$ has been shown to be associated with neurological change characterized by paraesthesia⁵. Four individuals in Jacareacanga, all fisheaters, either exceeded or approached this threshold, from a total sample of 25. Blood Hg levels in Jacareacanga were on average several times higher than in the other sites.

Environmental samples were also taken at each site. Mercury data showed good overall agreement between the Tapajós valley and the results previously recorded for the Madeira basin². Concentrations of Hg in floordust samples taken from gold trading houses in Itaituba and Crepuri showed massive pollution of the workplace; several samples exceeded 1% Hg and there was visible Hg in floor dust at two locations. There were elevated Hg levels in fish samples from Itaituba, Jacareacanga and Crepuri, ranging up to 2.6 mg kg^{-1} fresh weight. The WHO

provisional tolerable weekly intake for methyl mercury is 0.2 mg , which would be provided by a weekly intake of only 1 kg of about half the 52 samples taken.

There is an urgent need to establish and quantify exposure pathways and the health impact of mercury in fisheating and gold mining/processing populations. It is also imperative that

cleaner technologies for gold mining and processing are developed. A three-year project was started in January to address these issues, financed by the European Commission.

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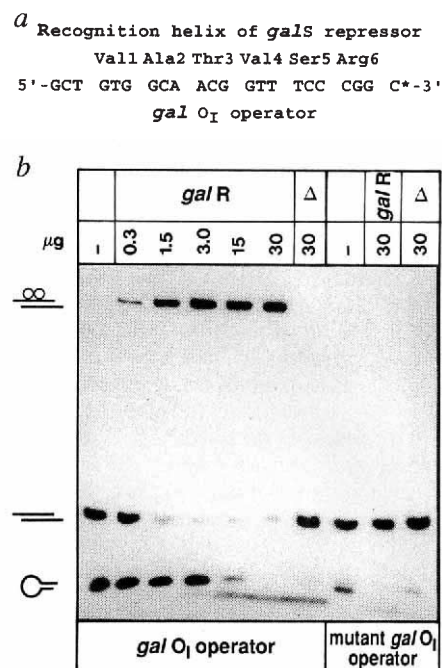
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DNA recognition and the code

SIR — The *galS* repressor¹ belongs to a large family of proteins that contain a helix–turn–helix motif. Weickert and Adhya¹ found that this repressor binds to an operator within its own coding sequence. We show here that this operator is identical to the DNA sequence coding for residues 1–6 of the recognition helix of this repressor. Our *in vitro* gel retardation experiments confirm the *in vivo* results of Weickert and Adhya, and show that *gal* repressor specifically retards a double-stranded 22-residue oligonucleotide encoding residues 1–6 of its recognition helix (see figure).

Does this phenomenon reflect a general property of the genetic code? Hickok and collaborators² have recently performed molecular modelling experiments to show that amino acids of the recognition helix of the glucocorticoid receptor can bind to their coding sequences. We have analysed six other real or model recognition helices in gel-shift experiments, but could not find



a, Sequences of the recognition helices of *galS* or *galR* repressor and *gal* operator *O*₁. b, Gel retardation assay³ with crude extract of *gal* repressor and *gal O*₁ operator or mutant *gal O*₁ operator. The mutant *gal O*₁ operator carries an A → C and a G → C exchange in codon 2 of the recognition helix. The triangle indicates crude extract prepared from the same *galR* host containing no *galR*-overproducing plasmid; symbols on the left indicate hairpin structure, free DNA and the protein–DNA complex (from bottom to top).

another example where there is any specific binding to the coding region (a list of sequences is available on request from the authors). The likelihood that the symmetry axis of *gal O*₁ is positioned as it is, is about 1 in 600, assuming it could be positioned anywhere upstream or downstream between base pairs 92 and 401 (the positions of *O*₂ and *O*₃ of the homologous *lac* system). The likelihood that the relevant base pairs of *gal O*₁ can encode residues 2–5 of the recognition helix of *galR* is less than 1 in 256 if we make the minimal assumption that the first position of the codons cannot be replaced by other base pairs. Thus the combined likelihood is about 1 in 10,000.

It remains to be seen whether our failure to find other examples indicates that the actual positioning of the *galS* operator is an accident of evolution.

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Steadying the rates

SIR — Wilson¹ used high-precision plate-rotation solutions for five plate pairs to test the astronomically calibrated timescale^{2,3} over the past 5.32 million years. Wilson based his analysis on the determination of total rotation poles and provided a confidence interval for spreading distance with the significance level better than 95%. He found that the use of the astronomical ages rather than those from the standard polarity timescale⁴ resulted in very small and physically realistic spreading rate variations. As a result, plate-motion rates of the NUVEL-1 global model⁵ need to be revised downwards by 4.5%, leaving a marginally significant difference of 1.5% with geodetic plate-tectonic rates averaged over years⁶. Wilson concluded that the errors in the astronomical calibration are no greater than 20,000 years and that the spreading rate can remain constant for several million years.

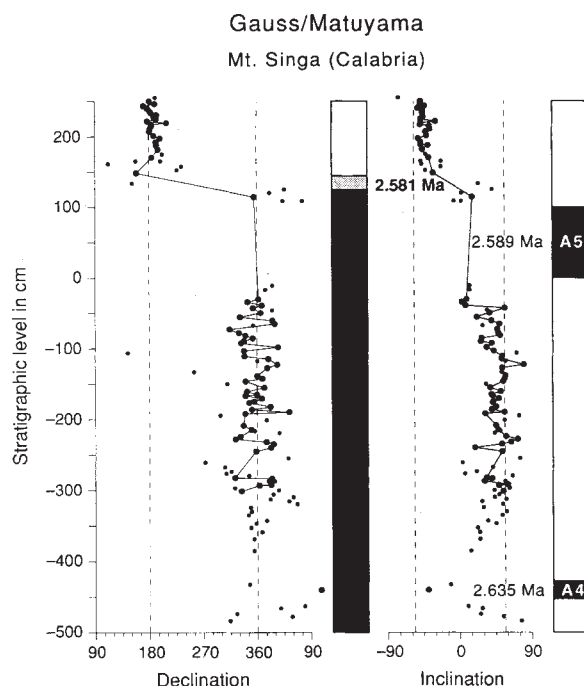
In four of the five plate pairs only the Gauss-Matuyama (GM) boundary shows a (reduced) distance that — within its confidence interval — significantly differs from the assumption of constant spreading. Wilson inferred minor refinements to the astronomical timescale and suggests that the age of 2.60 million years (Myr) ago² for the GM boundary appears too old by 10,000–20,000 years. Similarly, he suggested that the base of the Nunivak (4.62 Myr ago³) may be slightly too young, although uncertainties are larger.

Here we provide a refined age of the GM boundary which confirms Wilson's suggestion.

The astronomical ages reported by F.J.H.³ are based on earlier magnetostratigraphic studies^{7,8} of Pliocene–Pleistocene land sections in the central Mediterranean. All these reversals have subsequently been sampled and studied in detail (ref. 9, and our unpublished data), thereby providing their exact positions and hence more accurate ages. The detailed record of the lower Nunivak¹⁰ now provides a best age estimate of 4.618 Myr ago, the same as the earlier estimate of

4.62 Myr ago³. For the GM boundary, Wilson used the age of 2.60 Myr ago (from ref. 2), whereas F.J.H.³ gives two ages: the older age of 2.62 Myr ago is probably caused by delayed NRM acquisition¹⁰; the younger age of 2.59 Myr ago was based on the magnetostratigraphy of the Mounte Singa section⁷.

We have resampled the GM boundary in detail in this latter section. We applied progressive stepwise thermal demagnetization to all samples; the palaeomagnetic characteristics are very similar to those from the earlier magnetostrati-



The GM reversal record sampled in detail in the Mounte Singa section⁷ in southern Calabria. Characteristic remanent magnetization directions (declination and inclination) are derived from progressive stepwise thermal demagnetization; large (small) dots are reliable (less reliable, low intensity) directions. The polarity column (left) shows normal (black) and reversed (white) polarity; the shaded part denotes the GM transition interval. The lithological column denotes marine clays (white) and the sapropelitic layers⁷ (black). The mid-points of sapropels A4 and A5 have astronomically calibrated (4,000 year lagged) ages of 2.635 and 2.589 Myr ago, according to ref. 3. The average sedimentation rate of 10.7 cm per 1,000 years between the sapropels has been used to extrapolate an age of 2.581 Myr ago for the GM boundary at the 135-cm level.

graphic study⁷. The results (see figure) show that the GM boundary is at level 135 cm, above the top of sapropel A5 which has a midpoint (at 50 cm) with an age of 2.589 Myr ago³; the lower sapropel A4 has a midpoint (at -440 cm) with an age of 2.635 Myr ago³. The resulting average sedimentation rate of 10.7 cm per 1,000 years has been used to extrapolate an age of 2.581 Myr ago for the GM boundary. This new age shows that Wilson's analysis gives an accurate test of astronomical ages, and that his suggestion of a younger GM boundary is correct. Indeed, refinement of orbital dates steadies the rates⁶.

The slightly older astronomical age of 2.60 Myr ago of Shackleton *et al.*² is derived from correlation of the $\delta^{18}\text{O}$ record of the Ocean Drilling Program Site 677 to that from the Deep Sea Drilling Project (DSDP) Site 607, where the GM boundary falls within oxygen isotope stage 104 (ref. 11). The boundary appears to fall at slightly younger levels, however, in DSDP sites 609 and 552A, that is, in stage 103 (ref. 11). Evidently, this latter position is consistent with the boundary in the Mounte Singa section. The younger age is preferred, because sedimentary remanence mechanisms can make the reversal appear older, but not younger.

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Sea-level rise

SIR — Sahagian *et al.*¹ have assessed the contributions to global sea-level rise of groundwater withdrawal, surface-water diversion and land-use changes. One of their key conclusions is that these direct anthropogenic contributions account for 30% of the total observed sea-level rise this century. But their data actually account for only a 7% rise.

Sahagian *et al.* calculate that human activities are at the present time causing a sea-level rise of 0.54 mm per yr, and have caused a total sea-level rise since 1900 of 11.8 mm. For the average observed rise since 1900, they take a value of 1.75 mm per yr from the literature. It is indeed true that if one assumes a constant sea-level rise since 1900, which is open to question, a value of around 30% ($0.54/1.75=0.31$) for direct anthropogenic contributions to actual sea-level rise is obtained. But Sahagian *et al.* state that this value is valid for total sea-level rise during the twentieth century, which is incorrect. It should be 7% ($11.8/(94 \times 1.75)=0.07$). In any event, sea-level changes integrated over longer periods, for example over 1900–94, are more interesting than calculating the current rate of rise.

According to the numbers above, the ratio of the direct anthropogenic contributions to the observed sea-level rise has increased during the past 100 years up to the actual ratio of 0.31. Thus, would an integration over, for instance, the next 50 years point towards an important role of human activities in sea-level rise during that period? This seems not to be the case: Sahagian *et al.* compute a sea-level rise due to human activities in the next 50 years of 26.1 mm. The IPCC report² predicts a sea-level rise due to climate-related factors (thermal expansion of the oceans and volume change of land ice) of 210 mm, giving 11% for the contribution of human activities.

I also disagree with the statement that, finding 30% for the direct anthropogenic contributions to sea-level rise in the twentieth century, "the contributions of glacial melting and ocean thermal expansion are smaller than previously thought". No reference is given. According to the IPCC report², observed sea-level rise over the past 100 years is 15 ± 5 cm and the sum of estimates of the climate-related contributions is 10.5 ± 11 cm. Disregarding the uncertainties, these values allow for a contribution of exactly 30% by other factors than the climate-related ones. After reading Sahagian *et al.*, I understand that 7% can be attributed to groundwater withdrawal, surface-water diversion and land-use changes.

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SAHAGIAN *ET AL.* REPLY — Greuell suggests that past sea-level changes integrated over long periods of time are more interesting than actual rates. We disagree and maintain that the current rate of sea-level change induced by humans is the datum of greatest interest for comparison with climate-induced changes in sea level, because it provides the basis for extrapolation into the future. Our figure of 30% pertains to the present rate of rise caused by anthropogenic factors, compared with the total measured rate as averaged over twentieth-century tide gauge records.

There are two reasons that the current rate of anthropogenic sea-level rise is larger than the average rate throughout this century. First, the rate of anthropogenic contributions has increased throughout this century, apart from a temporary reduction due to major dam-building projects in the 1960s and 1970s. Second, the major dam-building activities reduced the twentieth-century average, but have now virtually ceased. The present rate of 0.54 mm per yr is thus not an average of all anthropogenic factors since 1960 because it does not include dams.

Indeed, this figure is a conservative underestimate, as the trend of increasing rates would suggest that the present rate is greater than the average over the past 34 years. Similarly, the present rate (0.54 mm per yr) provides a conservative basis for future extrapolation as it assumes that the anthropogenic contributions will cease the pattern of increase exhibited throughout the twentieth century.

Greuell cites an IPCC report² for future sea-level rise due to climate factors which is based on various models and predicts a much greater contribution in the future than has been observed in the past (or present). It follows that when compared this way, he obtains a low figure of 11% for the anthropogenic contribution. One could take into account projected increases in water needs of the growing human population and calculate a larger percentage. We are reluctant to do this because the uncertainties in local and global economics, development of arid and forested regions and many other factors make such projections unreliable.

Greuell makes an argument based on

"disregarding uncertainties" in the IPCC estimate² of climate-related contributions of 10.5 ± 11 cm. We feel that uncertainties which are larger than the magnitude of inferred sea-level rise cannot be disregarded, and that this figure cannot be used for reliable projections. Our efforts have been directed at identifying additional factors which must be considered before estimates of climate contributions to sea level can be deduced from measured sea-level rise.

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Pore region of K⁺ channel RACK1

SIR — Suzuki *et al.*¹ have published the sequence of a potassium channel involved in the secretion of K⁺ from the renal collecting duct. This channel is inhibited by protons, explaining the well-established competition between K⁺ and

hydropathy plot, and using the program CAMELEON (Oxford Molecular Ltd), would give the result shown in the bottom row of the sequence comparison below, Ala 181 being underlined in both top and bottom rows. This alignment also gives a

RACTK1 (residues 168–189)	WHMGFFLSSILP A SGKLVSTTA
IRK1 (132–153) (ref. 2)	AFLFSIETQTTIGYGFRVCTDE
GIRK1 (133–154) (ref. 3)	AFLFFIETEATIGYGYRYITDK
ROMK1 (131–152) (ref. 4)	AFLFSLETQVTIGYGRFVTEQ
RACTK1 (modified alignment; 181–202)	ASGKLVSTTATIFFGSDLNIAG

H⁺, and by ATP¹. The authors offered a suggested alignment of their channel sequence with that of other related channels from the family containing the inward rectifiers IRK1 (ref. 2) and GIRK1 (ref. 3), and another channel from renal tubule, ROMK1 (ref. 4). These channels apparently have in common the possession of only two membrane-spanning α -helices.

So far as the so-called H5 or P (pore-forming) region of the channel is concerned, the alignment suggested by Suzuki *et al.*¹ is as shown above.

But an alignment more consistent with what is known of this region in other K⁺ channels is achieved if the constraint is imposed that the motif TXXT, common also among voltage-gated K⁺ channels (see, for example, ref. 3), is conserved. Carrying out an alignment with this constraint, informed by a Kyte–Doolittle

conserved Gly (position 195) following Phe in place of Tyr in other K⁺ channels. Note that the ion channel associated with the *ether-a-go-go* mutation of *Drosophila* has Phe in place of Tyr in an equivalent position⁵, but this channel permits Ca²⁺ as well as K⁺ permeance⁶. Our proposed alignment suggests that RACK1 is more closely related to other channel types than proposed by Suzuki *et al.*¹.

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If this be magic . . .

A. Rupert Hall

Science and the Secrets of Nature: Books of Secrets in Medieval and Early Modern Culture. By W. Eamon. Princeton University Press: 1994. Pp. 490. £38.50, \$49.50.

MANY years ago, my pupil Elizabeth Ryman earned herself a few pounds by sending to the now defunct magazine *Lilliput* (which welcomed curiosities) a page from Giambattista della Porta's *Natural Magick* (1558), perhaps that containing recipes for whitening the face. "According to Della Porta", writes William Eamon, "natural magic was a science of the extraordinary [explaining] the exceptional, the unusual and the miraculous"; it also purveyed cosmetic tips and wrinkles. Like many other Renaissance scholars, della Porta had a basic principle: he believed that the power and secrets of nature were to be found not in matter but in morphology. Form accounted for everything. The basis of true knowledge was understanding the relationship between forms (as we might say, structures), especially between likes and unlikes. If the analogy between the walnut in its shell and the brain in its skull taught a lesson to physicians, equally the sage must note that polar opposites are "universal principles of intelligibility. . . . A well-ordered commonwealth implied the existence of a world upside-down." At any moment one might tumble from this world into a looking-glass one.

Another delusion of respectable antiquity (with which Eamon opens his book) was that the highest knowledge can be known only to an élite, not because it demands big brains but because it can be acquired only by patient adepts who have absorbed it through years of study. A famous exposition of such esoteric learning is the pseudo-Aristotelian *Secret of Secrets* — in fact borrowing much from the Ismaili sect of Islam — perhaps the most popular book of the Middle Ages, the great inspiration of Roger Bacon (among others) for whom it stood "midway between the Hebrew prophets and the Latin Christians".

Another popular book of secrets, falsely attributed to Albertus Magnus, was censured by Bacon as magical, although it employed only natural means (such as a goats' hair to bring about pregnancy)

rather than the invocation of demons. Many such odd practices seemed as absurd to mediaeval schoolmen as to ourselves, although they rejected them less because of their lack of empirical success than because they sprang from the popular mind as opposed to the learned texts of antiquity.



The Alchemist Experiment Takes Fire by Hendrik Heerschoop.

Magic was a supreme secrecy always officially condemned; the secrets of craftsmanship and especially of new inventions that began to be divulged in the later Middle Ages were of a different kind. 'Theophilus' linked monastic crafts to the service of God in *On Diverse Arts* (twelfth century) while in 1335 Guido da Vigevano suggested new military devices to aid a proposed crusade. In the fifteenth century such practitioners as Mariano Taccola and Francesco di Giorgia Martini committed their skills extensively to paper, although they were aware of the risk of "giving up the fruit of one's ingenuity". With the introduction of printing and greater literacy in Europe, in Germany the *Kunstbüchlein* (from 1531), and in Italy the *House of Recipes* (1525, a prototype Mrs Beaton), prepared the way for the magisterial works of technological description by Biringuccio, Agricola and Ercker. Meanwhile, at Venice in 1535, the pseudony-

mous 'Alexis of Piedmont' published the most successful of all books of secrets, reaching 70 multilingual editions in half a century. 'Alexis' gave roughly equal numbers of medical, domestic and technical recipes, originally about 350 in all. He taught how to treat piles and make artificial vermilion. Printers of chapbooks, Italian *ciarlatani* and vendors of gold bricks and gilded pills were not slow to profit from his openness, and from the books of della Porta, Ruscelli and others, so that within half a century 'secrets' could be bought retail in every market. Was this a profanation of high mysteries? Or was the carrying of a weasel's heart to prevent the bite of bedbugs always rather a joke among the literate, and the codex of 'secrets' the *Playmonk* of the priory?

Eamon gives a rich and lively account of authors and writings that were always unacademic, unscrupulous, unprofessional, turbulent and unsettled: that is to say, an account of the popular or seamy side of medicine and natural knowledge in mediaeval and early modern times. In 'books of secrets' the range of topics covered was far wider than that of academic learning; it followed the spirit of 'do it yourself' and of 'experiment' ('try anything once'). It demanded belief not on grounds of reason or antique authority but by making the simple empiric's claim: "Believe me, it works"; this validated the quenching of iron in juice of radishes, excising a swollen spleen or boiling puppies and earthworms together to make an ointment. More dubiously, perhaps, Eamon relates the tradition of these 'secrets' of nature to the seventeenth-century search for the mechanisms of nature, certainly linked as they are by such authors as Sir Kenelm Digby (omitted here). By then the 'secrets' had become the 'vulgar errors' exposed by Dr Thomas Browne and others, but their activist, operative tradition may have contributed to the substitution of experimental science for purely rational science.

This is a book of many unusual topics: the careers of della Porta and Leonardo Fioravanti, the south Italian academies of the sixteenth century, the weird world of Early English Paracelsans and *Books of Conceits*. Eamon is very learned and writes eloquently. □

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Tracks in time

Douglas Palmer

The Palaeobiology of Trace Fossils.

Edited by Stephen K. Donovan. Wiley: 1994. Pp. 308. £39.95, \$63.95.

THE study of trace fossils has come a long way from the odd photograph of a chubby baby sitting in one of the large sauropod dinosaur footprints at the Paluxy River site in Texas (as reproduced in A. S. Romer's *Man and the Vertebrates*). Today, television has led us to expect a more dynamic approach. I remember McNeil Alexander strutting his stuff complete with stetson in a Second World War amphibious landing vehicle, speeding across the sands of Morecambe Bay, England, with pony and rider alongside changing gait and stride length with increasing speed. The purpose of all this was to illustrate a method of calculating the speed of dinosaur locomotion from the spacing of their footprints as recorded by trace fossils in sedimentary rocks.

But I exaggerate slightly. As Adrian Hunt and his co-authors of chapter 9, "The palaeobiology of vertebrate coprolites", remind us, trace fossils have a pedigree stretching back, as so often in the 'natural sciences', to nineteenth-century clergymen such as Dean Buckland. As early as 1823, he used the evidence of a very particular type of trace fossil, namely coprolites (faeces), to interpret a Pleistocene cave as a hyena den. Consequently, he was able to show a direct

comparison between modern hyena scat and the fossil coprolites and bone damage with that produced by a circus hyena.

Unfortunately, the limitations of Buckland's neontological methodology are even more severe for the study of trace fossils than they are for palaeontology in general. The very nature of trace fossils and their preservation history is such that rarely do we know 'who dunnit'. So the question of taxonomy is somewhat problematic to say the least, but is confronted by Ron Pickerill in the opening chapter of this stimulating new book.

Stephen Donovan has brought together a wide range of essays with a focus on different aspects of the evolution and palaeobiology of trace fossils, from the "Functional morphology of boring and burrowing invertebrates" by Enrico Savazzi, elegantly illustrated and with a warning note about the limitations of this kind of analysis, to the now familiar territory of the 'dino' trackers. Martin Lockley and colleagues discuss vertebrate tracks in the context of the ichnofacies concept. This builds on a recent spate of books (such as D. D. Gillette and M. J. Lockley's *Dinosaur Tracks and Traces* (Cambridge University Press, 1989) and Tony Thulborn's *Dinosaur Tracks* (Chapman and Hall, 1990)) reflecting something of a renaissance in the study of dinosaur footprints, which are probably more common than dinosaur bones. So much so that it is possible to extend Dolf Seilacher's concept of recurring assemblages of similar trace fossils associated with particular sediment types (facies, and hence ichnofacies) to recurring collections of dinosaur footprints, although the latter are inevitably more restricted in their temporal range.

Donovan himself contributes a timely essay on insects and other arthropods as trace-makers in non-marine environments. This area is attracting a lot of attention, and even since the publication of this book there is new evidence from the British Lake District (E. W. Johnson *et al.*, *Geological Magazine* **131**, 395; 1994) that continental environments were probably colonized much earlier than was previously thought. And the critical evidence is recorded by trace fossils made in shallow freshwater volcanogenic sediments from the Borrowdale Volcanic Group by myriapod-like arthropods as long ago as Ordovician times.

The contribution that the study of trace fossils has already made and will undoubtedly continue to make far outweighs semantic problems. This book is a useful and stimulating collection of essays that should be read by anyone interested in new ways of understanding life of the deep past.

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Cosmic integers

John Polkinghorne

Is There A Universe? By Stanley L. Jaki.

Liverpool University Press, UK: 1994. Pp. 137. £16.50 (hbk); £8.95 (pbk).

COSMOLOGY is very much part of the current scientific agenda and its popularizations find their place in the small change of educated conversation. It is surprising to be reminded, as we are by Stanley Jaki, of how late a development in thought the subject is and how many people previously resisted talk of the Universe, understood as the totality of things. Jaki sees Einstein's relativistic cosmology of 1917 as a key development representing the first respectable way of speaking of a cosmos and also avoiding the paradoxes that Newtonian gravitation brought to the concept. Yet he regards science as of comparatively little use in addressing the question of his title: "Such an entity [the Universe], if it is to be spotted, calls for philosophical eyes, eyes usually very weak in scientists". Questions of realism or idealism, being or becoming, the existence of an actual realizable infinity, are all held to lie behind the question.

Jaki is very learned and there is much interesting material in this book, which is based on lectures given at the University of Liverpool. Jaki is also very polemical, and scornful of those whose ideas do not precisely match his own concepts, derived from the philosophy and theology of Thomas Aquinas. He is a chronicler of intellectual endeavour, but he lacks a historian's sympathy for the perspectives of the past. All is illuminated by hindsight and all must pass through the sieve of Thomist orthodoxy. Many celebrated figures incur Jaki's wrath: Plato, Kant and, archvillain of them all and no doubt consigned to the lowest circle of the Jakian hell, Niels Bohr. When we come to Jaki's own answers to his question, they prove to be rather odd, and to me unconvincing. Countability is the key — "a property absolutely indispensable for the purposes of science" — and the integers enable Jaki to give a positive answer to his own question. It seems that for him, existence is enumeration.

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■ *The Fermi Solution: Reflections on the Meaning of Physics* by Hans Christian von Baeyer has just been issued by Penguin in the UK (£6.99 pbk); published last year by Random House in the USA. The essays first appeared in *The Sciences and Discover* and are by the author of the highly praised *Taming the Atom* (see *Nature* **361**, 215; 1993).

New in paperback

Evolution and Extinction edited by W. G. Chaloner and A. Hallam. Cambridge University Press, £22.95, \$37.95. Well-presented proceedings of a joint symposium of the Royal Society of London and the Linnean Society held in 1989. Contributors include J. Maynard Smith, A. K. Knoll, M. J. Benton, D. M. Raup and J. M. Diamond.

Visions of Caliban: On Chimpanzees and People by Dale Peterson and Jane Goodall. Houghton Mifflin, \$12.95.

"Beautifully written, easily read and ethically challenging — it might just become primatology's *Silent Spring*" (Alison Jolly, *Nature* **362**, 678; 1993).

Fuzzy Thinking: The New Science of Fuzzy Logic by Bart Kosko. A popular — and slightly breathless — account by a pioneer in the field. Hyperion, \$12.95.

Germs, Seeds and Animals: Studies in Ecological History by Alfred W. Crosby. M. E. Sharpe, £14. A collection of previously published essays in which a noted historian discusses the biological and ecological consequences of European expansion since 1492. Fascinating and entertaining reading.

Environmental stewardship

R. J. Berry

Caring for Creation: An Ecumenical Approach to the Environmental Crisis. By Max Oelschlaeger. Yale University Press: 1994. Pp. 285. \$35, £22.50.

The Earth, Humanity and God. By Colin A. Russell. UCL Press: 1994. Pp. 193. £30 (hbk); £9.95 (pbk).

MAX Oelschlaeger is an environmental philosopher at the University of North Texas, best known for his valuable study of the *Idea of Wilderness* (Yale University Press, 1991). He begins his new book with a profession of conversion: that he had been wrong to trust in the "experts" who claimed to be able to "manage planet Earth" and that he had been mispersuaded by Lynn White's influential essay on "the historical roots of our ecological crisis", published in *Science* in 1967, about the culpability of Judaeo-Christianity for environmental problems. He is encouraged by international agreements on environment cooperation, but judges that politicians are too narrow-minded and technologists too powerless to take effective action. (He quotes the Agenda of Science for Environment and Development into the Twenty-First Century (ASCEND) Conference of the International Council of Scientific Unions (IUCN) in Vienna, which shaped the Agenda 21 at the United Nations Conference on Environment and Development in 1992, and the joint declaration of the Royal Society of London and the US National Academy of Science, which urged action on such problems as population, climate warming and biodiversity.)

Oelschlaeger's indictment of the environmental record of politicians is well worn, but needs reading into the record of all aspirants. For example: "issues of ecological consequence are not parceled out in terms of political office" (identified as lasting 4 ± 2 years); the 1980s represented "the Reagan antienvironmental revolution"; gross national product is "a truncated, obsolete and increasingly unworkable measure of the economy"; and "competition undercuts voluntary cooperation across firms". He quotes the US vice-president, Al Gore: "emphasis on the rights of the individual must be accompanied by a deeper understanding of the responsibilities to the community that every individual must accept if the community is to have any organizing principle at all." His conclusion is that only religion offers a viable and neutral forum to produce the necessary commitments on lifestyle, resource use and discipline.

Although he acknowledges his debt to



THE discovery in 1991 of this mummified Neolithic man in an alpine glacier on the Austrian-Italian border attracted widespread attention. But the delay in publication of detailed information about the body and its associated finds led to nagging doubts about the iceman's authenticity (see Commentary in *Nature* 362, 11; 1993). These rumours are now laid to rest with the results of DNA tests, reported in last week's issue of *Science*, as well as with the recent publication of *The Man in the Ice* by Konrad Spindler, one of the first archaeologists to investigate the body. The book, from which this picture is taken, claims to provide "the fullest picture yet" of the discovery. Weidenfeld and Nicolson, £18.99.

Francis Schaeffer's *Pollution and the Death of Man* (Hodder and Stoughton, 1970), which was itself a response to White's article, Oelschlaeger's ecumenical vision is almost entirely atheological. He speaks about skyhooks ("privileged metaphysical claims of religions") and religious toeholds (an acknowledgement of "a sacred canopy or ultimate belief that frames the world"), but he is really concerned with a medium for non-sectarian dialogue. This is an odd claim to make for religions, but possible in the North American ethos, which is Oelschlaeger's specific context. His argument is that four institutions (state, corporation, university and church) "shape social and natural ecology", but only the church is outside the competitive grudges stimulated by monetarization. Furthermore, the church is involved because "our ethical and political lives are collapsing as [the result of] the unbridled pursuit of a narrowly defined and scientifically tenuous conception of economic success".

There are people who will disagree with this diagnosis and prognosis about the structure of society, but they are mainly those outside the scientific community or who have a vested interest in the status quo. The proposal that the church provides a way forward is even more contentious; here it has to be recognized that Oelschlaeger's prescription is specifically directed towards the North American

situation. But his general point about the gap between environmental problem and environmental response is wholly valid. (I am referring here to rationally directed scientific response, rather than the excited, sincere but sadly random reaction of 'concerned citizens'.) An excellent cautionary tale (not recorded by Oelschlaeger) is the *World Conservation Strategy* (International Union for the Conservation of Nature/Worldwide Fund for Nature/United Nations Environment Programme). This was a clearly argued case for sustainable development through careful conservation, but fell heavily into the Enlightenment fallacy that rationality is inseparable from response. Through the perspicacity of Max Nicholson, the *Conservation and Development Programme for the UK* (Kogan Page, 1983) contained a substantial section on ethics, emphasizing the importance of personal and corporate value as an essential component and stimulus of commitment. In 1986, the IUCN (the main author of the strategy) recognized the omission in the original document, and set up an ethics working group. This contributed significantly to the revised strategy, *Caring for the Earth* (1991).

Other agencies have responded similarly. The G7 nations held a conference on environmental ethics in 1989 and agreed on the need for responsible stewardship; the following year they produced a "Code

of Environmental Practice" involving a series of moral responses and practical obligations. The UK white paper (policy document) *This Common Inheritance* (HMSO, 1990) began with a statement of general principle: "We have a moral duty to look after our planet and hand it on in good order to future generations. . . . We must not sacrifice our future well-being for short-term gains, nor pile up environmental debts which will burden our children." The policy statement *Sustainable Development: The UK Strategy* (HMSO, 1994) reaffirmed this commitment.

There are other examples. The point is that Oelschlaeger's thesis is not original, but needs continual reiteration. I hope that North Americans read it and heed his message, and that rational beings elsewhere heed it also and do not get distracted by the North American framework.

The Earth, Humanity and God by Colin Russell, a professional science historian, is explicitly theological and splendidly conservative. By this I mean that he shows

the consistency and coherence of the responsibility doctrine embodied in Judaeo-Christian teaching with a proper application of conservation measures. He argues that both conventional reductionist science and postmodern mystical claims of the Earth as alive are inadequate by themselves, and that a third approach based on "stewardship" offers the best prospect for the future.

The book is the fruit of a lecture series given by Russell in Cambridge, England, in 1993 under the auspices of the Templeton Foundation. If other series attain this level, we shall be enriched. There will be readers who do not share Russell's Christian commitment, but they will do well to take seriously his account of the interplay of faith, reason and fear; none of us is exempt, even if our faith is in human rationality or nihilism rather than a conventional God. □

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from coal through hydrogenation.

But bombing and destruction left the chemical industry at a standstill after the war. The Allies had achieved their goal of breaking up I. G. Farben into smaller companies, a process that was the subject of Stokes's first book *Divide and Prosper*. The sequel is even more authoritative; Stokes demonstrates great command over his sources.

By the 1960s, coal had been largely abandoned as a feedstock by most West German companies in favour of the cheaper and better US petroleum methods. But Stokes provides one example of a small company, Bergkamen, that continued to use the Fischer-Tropsch process until well after the war. Like BASF and Bayer, which had 50–50 partnerships with Shell and BP, respectively, Bergkamen eventually abandoned any hope of economic self-sufficiency and began to embrace international trade and to use petroleum as a feedstock.

A topic Stokes only touches on is the comparison with developments in East Germany. Material became available to him only after the book was completed and a more detailed account is planned for another volume. The comparison, however, will be illuminating. It seems that although East Germany recognized the importance of petroleum, it committed itself more to coal-based chemistry, falling behind the West for many reasons, including the lack of foreign exchange, old-fashioned equipment, the shortage of employees as a result of emigration to the West and ties to the Soviet economy. All this requires another book to complement this fine achievement in scholarly analysis. □

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Fuelling the engines of growth

Kristie Macrakis

Opting for Oil: The Political Economy of Technological Change in the West German Chemical Industry, 1945–1961. By Raymond G. Stokes. Cambridge University Press: 1994. Pp. 259. £30, \$49.95.

VISITORS to East Germany in the early 1980s were often struck by the pungent smell of coal in the air. Used as a feedstock, or primary starting material, coal had there remained almost synonymous with industry — but not so in West Germany.

Raymond G. Stokes's finely crafted book is one long argument supporting the claim that it was, in part, the transition from a traditional coal-based industry to a modern petroleum-based industry that allowed West Germany to compete in world markets. The transition helped the country to achieve the economic miracle of the 1950s that brought success during the 1960s and later.

The petroleum-based chemical industry spawned technological developments that transformed the world and vastly improved standards of living: after the Second World War, plastics began to dominate the world market, and polyethylene, widely used in wire insulation, containers and packaging, became one of the most important products to be manufactured from petroleum. But the nitty-gritty of these products is not the author's main concern. Rather, Stokes gives a highly interpretative historical account that focuses on business, technol-

ogy and Germany as a whole. It is mostly organized chronologically. After several background chapters (1860–1945), he introduces his analysis of technological change and transfer, and provides case studies of representative companies to show the alternative paths taken towards prosperity. The transition to oil was not uniform in all industries and involved a gradual change in cultural attitudes.

Germany has a venerable tradition of excellence in the chemical industry. Dyes, pharmaceuticals and synthetic fuels have long been associated with a country backed by a skilled and disciplined workforce. By the end of the nineteenth century, the organic chemical industry had become an engine for economic growth and a source of political power. German companies soon dominated and monopolized world markets; in 1913, 88 per cent of the world's dyestuffs came from Germany.

BASF, Hoechst, Bosch and Bayer were all founded either on the eve of German unification (1871) or in its aftermath. By the beginning of the Weimar period, I. G. Farben was formed as an umbrella organization for these companies — and in unity lay strength. New technologies were developed during the 1920s and 1930s to harvest the fruits of raw materials available to Germany, and during the period of National Socialism, a state-sanctioned policy of economic self-sufficiency accelerated the need for such technologies. One of these methods was the Fischer-Tropsch process, implemented by the Nazis to manufacture liquid fuels

Recent textbooks

The Invertebrates: A New Synthesis by R. S. K. Barnes, P. Calow and P. J. W. Olive (2nd edn). Blackwell Scientific, £47.50 (hbk), £19.95 (pbk). "Students will like this book. It deserves to succeed", wrote A. Brafield of the first edition (*Nature* **338**, 185; 1989).

Biology of Amphibians by William E. Duellman and Linda Trueb (2nd edn). Johns Hopkins University Press, £33 (pbk). In a review of the first edition, T. Halliday wrote that the text was "clear, concise and richly illustrated . . . likely to be an important source of reference" (*Nature* **319**, 364; 1986).

The Cell Cycle: An Introduction by Andrew Murray and Tim Hunt. Oxford University Press, £15.95 (pbk).

Modern Parasitology edited by F. E. G. Cox (2nd edn). Blackwell Scientific, £18.95 (pbk). Greatly revised, with a new chapter on molecular biology.

Structural determinants for activation of the α -subunit of a heterotrimeric G protein

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The 1.8 Å crystal structure of transducin α -GDP, when compared to that of the activated complex with GTP- γ S, reveals the nature of the conformational changes that occur on activation of a heterotrimeric G-protein α -subunit. Structural changes initiated by direct contacts with the terminal phosphate of GTP propagate to regions that have been implicated in effector activation. The changes are distinct from those observed in other members of the GTPase superfamily.

HETEROTRIMERIC guanine-nucleotide-binding proteins (G proteins; subunits $\alpha\beta\gamma$) function as molecular switches in a diverse set of signalling pathways by coupling seven-transmembrane-helix receptors (heptahelical receptors) to specific intracellular effectors. The transduction of signals depends on the ability of the α -subunits to cycle between a resting (GDP-bound) conformation primed for interaction with agonist-stimulated receptors and an active (GTP-bound) conformation capable of activating or inhibiting a variety of downstream effectors including enzymes as well as ion channels^{1,2}. In response to extracellular signals (such as hormones, neurotransmitters, opiates, odorants and light), heptahelical receptors catalyse the exchange of GTP for GDP, resulting in an active conformation with high affinity for specific downstream effectors and greatly reduced affinity for the $\beta\gamma$ subunits³.

One of the best characterized heterotrimeric G-protein-coupled systems is the visual cascade of retinal rod outer segments⁴⁻⁶. Here, signal transduction begins with the absorption of a photon by the 11-*cis*-retinal chromophore of the photoreceptor rhodopsin (Rh). Rapid photoisomerization to all-*trans*-retinal triggers a series of structural changes that lead to the formation of the activated intermediate meta-rhodopsin II (Rh*). Rh* binds the heterotrimeric GDP-bound form of transducin ($G_{ta}\beta\gamma$) and catalyses the exchange of GTP for GDP, resulting in the dissociation of $G_{ta}\beta\gamma$ into G_{ta} ·GTP and $G_{i\beta\gamma}$. G_{ta} ·GTP, in turn, binds to and activates a potent cyclic GMP phosphodiesterase (PDE; subunits $\alpha\beta\gamma_2$) by displacing its inhibitory γ -subunits. The resulting decrease in second messenger cGMP concentration causes cation-specific cGMP-gated channels to close, leading to hyperpolarization of the rod outer segment membrane. As a result of an intrinsic GTPase activity, G_{ta} is inactivated by hydrolysis of GTP to GDP, returning the system to its resting state.

Heterotrimeric G proteins are members of a large superfamily of GTPases which mediate diverse processes such as cellular signalling, protein synthesis, vesicular trafficking and synaptic fusion⁷. Crystal structures of the GDP- and GTP-bound forms have been reported for two members of the GTPase superfamily, the monomeric G protein p21^{ras} (Ras)⁸⁻¹⁰ and the bacterial elongation factor EF-Tu^{11,12,44}. In Ras, the conformational differences are restricted to the two regions of the protein that contact the γ -phosphate of GTP. In EF-Tu, by contrast, nucleotide exchange is accompanied by a large domain rearrangement.

To establish the nature of the conformational change induced by the exchange of GTP for GDP and the switch mechanism by which the presence or absence of the γ -phosphate defines the 'active' or 'inactive' state of a G_{ta} subunit, it is necessary to compare the crystal structures of the GDP- and GTP-bound forms. We recently determined the structure of the active conformation of transducin- α complexed with a non-hydrolysable GTP analogue (G_{ta} ·GTP- γ S)¹³. We now report the crystal structure of the inactive form, transducin- α complexed with GDP (G_{ta} ·GDP). The structures of both the active and inactive forms have been refined to a resolution of 1.8 Å, enabling us to describe in accurate detail the nature and mechanism of the conformational switch in a heterotrimeric G-protein α -subunit and the functional implications for interaction with other components of the signalling cascade.

Structure determination and refinement

A 325-amino-acid proteolytic fragment (lacking the first 25 amino acids) of bovine retinal rod outer segment G_{ta} , complexed with either GDP or GTP- γ S and suitable for crystallization, was prepared by limit digestion with a lysine-specific protease, endoproteinase lysC¹⁴. To prevent additional cleavage during limit digestion, G_{ta} ·GDP was reversibly activated with aluminium fluoride¹⁵ which was subsequently removed by ultrafiltration. Crystals of G_{ta} ·GDP in the orthorhombic space group *I*222 were grown at 4 °C from sodium 2-(*N*-morpholino)ethanesulphonate(MES)-buffered solutions (pH 6.0) containing 10% polyethylene glycol-8000 (PEG 8000), 10% glycerol, and 0.2 M, MgCl₂. Monoclinic crystals of G_{ta} ·GTP- γ S (space group *P*2₁; 3 complexes per asymmetric unit) were grown as described¹³.

The crystal structure of G_{ta} ·GDP was solved at 4 Å by molecular replacement using the G_{ta} ·GTP- γ S structure refined at 2.2 Å resolution¹³ as a search model (Table 1). Both structures have been independently refined with simulated annealing against data which are >90% complete from 8.0 to 1.8 Å resolution (Table 1). The present model for G_{ta} ·GDP includes 314 residues from 27–340, a GDP molecule, a Mg²⁺ ion, 339 water molecules and has a final *R*-value of 21.1% for all data (18.7% for data >2 σ) and a free *R*-value of 23.6% (21.1% for data >2 σ) for a randomly selected subset (10%) of the data omitted before the start of refinement. An example of the $2F_o - F_c$ electron density map is shown in Fig. 2c. Intensities for G_{ta} ·GTP- γ S were collected and the refinement extended from the 2.2 Å resolution limit reported previously to the present resolution of 1.8 Å (Table 1). The current model for G_{ta} ·GTP- γ S, which covers all three complexes in the asymmetric unit, includes 952 residues (26–342 for protomers a and b; 26–343 for protomer c), three

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GTP- γ S molecules, three Mg^{2+} ions, 1,226 water molecules and has a final R -value of 22.9% for all data (21.1% for data $>2\sigma$), and a free R -value of 27.6% (25.8 for data $>2\sigma$). Both structures have excellent stereochemistry and ϕ - ψ angles within 10° of allowed regions of the Ramachandran plot. Procedural details of the structure solution and refinement are given in the legend of Table 1.

Overall structure and nucleotide binding

The fold of $G_{i\alpha}$, whether complexed with GDP or GTP- γ S, consists of two domains (Fig. 1), a GTPase domain common to members of the GTPase superfamily and an α -helical domain

unique to the highly homologous family of heterotrimeric G-proteins¹³. The GTPase domain consists of five helices ($\alpha 1$ – $\alpha 5$) surrounding a six-stranded β -sheet ($\beta 1$ – $\beta 6$) with five strands running parallel and one ($\beta 2$) running antiparallel to the others. In contrast to Ras and EF-Tu, the second of the five helices in $G_{i\alpha}$ ($\alpha 2$) is a 3_{10} helix rather than an α -helix. The helical domain has an entirely α -helical secondary structure with one long central helix (αA) surrounded by five shorter helices (αB – αF) and is linked to the GTPase domain by two extended strands, linker 1 (54–58) and linker 2 (173–179). Between these two domains lies a deep cleft within which the nucleotide is tightly bound.

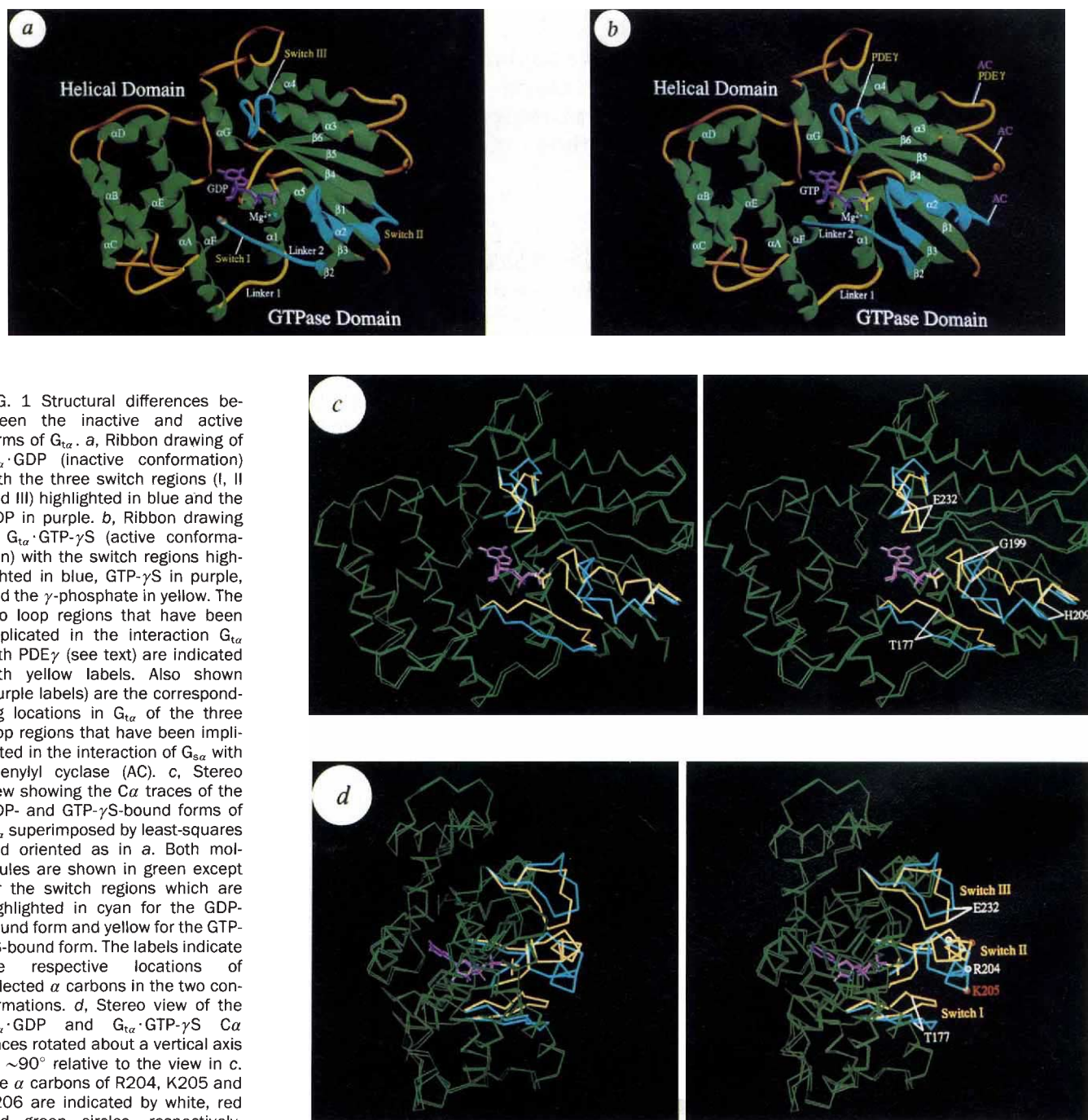


FIG. 1 Structural differences between the inactive and active forms of $G_{i\alpha}$. **a**, Ribbon drawing of $G_{i\alpha}$ ·GDP (inactive conformation) with the three switch regions (I, II and III) highlighted in blue and the GDP in purple. **b**, Ribbon drawing of $G_{i\alpha}$ ·GTP- γ S (active conformation) with the switch regions highlighted in blue, GTP- γ S in purple, and the γ -phosphate in yellow. The two loop regions that have been implicated in the interaction $G_{i\alpha}$ with PDE γ (see text) are indicated with yellow labels. Also shown (purple labels) are the corresponding locations in $G_{i\alpha}$ of the three loop regions that have been implicated in the interaction of $G_{s\alpha}$ with adenylyl cyclase (AC). **c**, Stereo view showing the $C\alpha$ traces of the GDP- and GTP- γ S-bound forms of $G_{i\alpha}$ superimposed by least-squares and oriented as in **a**. Both molecules are shown in green except for the switch regions which are highlighted in cyan for the GDP-bound form and yellow for the GTP- γ S-bound form. The labels indicate the respective locations of selected α carbons in the two conformations. **d**, Stereo view of the $G_{i\alpha}$ ·GDP and $G_{i\alpha}$ ·GTP- γ S $C\alpha$ traces rotated about a vertical axis by $\sim 90^\circ$ relative to the view in **c**. The α carbons of R204, K205 and K206 are indicated by white, red and green circles, respectively. Note the $\sim 90^\circ$ rotation of these residues on going from the inactive (cyan) to the active (yellow) conformation. **e**, Primary sequence alignment. Residues in the three switch regions are highlighted in light blue. Residues involved in conserved contacts to the γ -phosphate and switch II region are indicated by blue

and red boxes, respectively. The corresponding secondary structure for the $G_{i\alpha}$ ·GDP is shown below the aligned sequences. Sources of sequence information: bovine $G_{i\alpha}$ ³², bovine $G_{s\alpha}$ ³³, bovine $G_{i\alpha}$ ³⁴, bovine $G_{o\alpha}$ ³⁵, mouse $G_{q\alpha}$ ³⁶, human $G_{z\alpha}$ ³⁷, yeast $G_{\alpha 1}$ ³⁸, p21 Ras³⁹ and EF-TU⁴⁰.

Noel *et al.*¹³ suggested that the presence of the helical domain is the barrier that accounts for the slow rate of intrinsic nucleotide exchange ($\sim 10^{-3} \text{ min}^{-1}$ at 30 °C for G_{12a} (ref. 41)), allowing it to be controlled by the catalytic action of an activated receptor. In heterotrimeric G proteins, GDP release is independent of Mg²⁺ concentration⁴², whereas in Ras which lacks the helical domain, GDP release is highly dependent on Mg²⁺ concentration⁴³ (500-fold faster in nM than in mM Mg²⁺).

In addition to the bound nucleotide, both structures show clear density for a Mg^{2+} ion with six ligands coordinated in

octahedral geometry. In the $G_{1\alpha} \cdot \text{GTP} \cdot \gamma\text{S}$ structure, the nucleotide is essentially occluded from the bulk solvent and the Mg^{2+} ion coordinates oxygens from the β - and γ -phosphates, the side chains of Ser 43 and Thr 177, and two water molecules¹³. In the $G_{1\alpha} \cdot \text{GDP}$ structure, however, two of the ligands, the γ -phosphate oxygen and the side-chain oxygen of Thr 177, are replaced by water molecules. In addition, both linker 2 and the $\beta 3\text{-}\alpha 2$ loop are displaced from their respective locations in the $G_{1\alpha} \cdot \text{GTP} \cdot \gamma\text{S}$ structure, resulting in greater solvent exposure in the vicinity of the β -phosphate and Mg^{2+} ion. The other

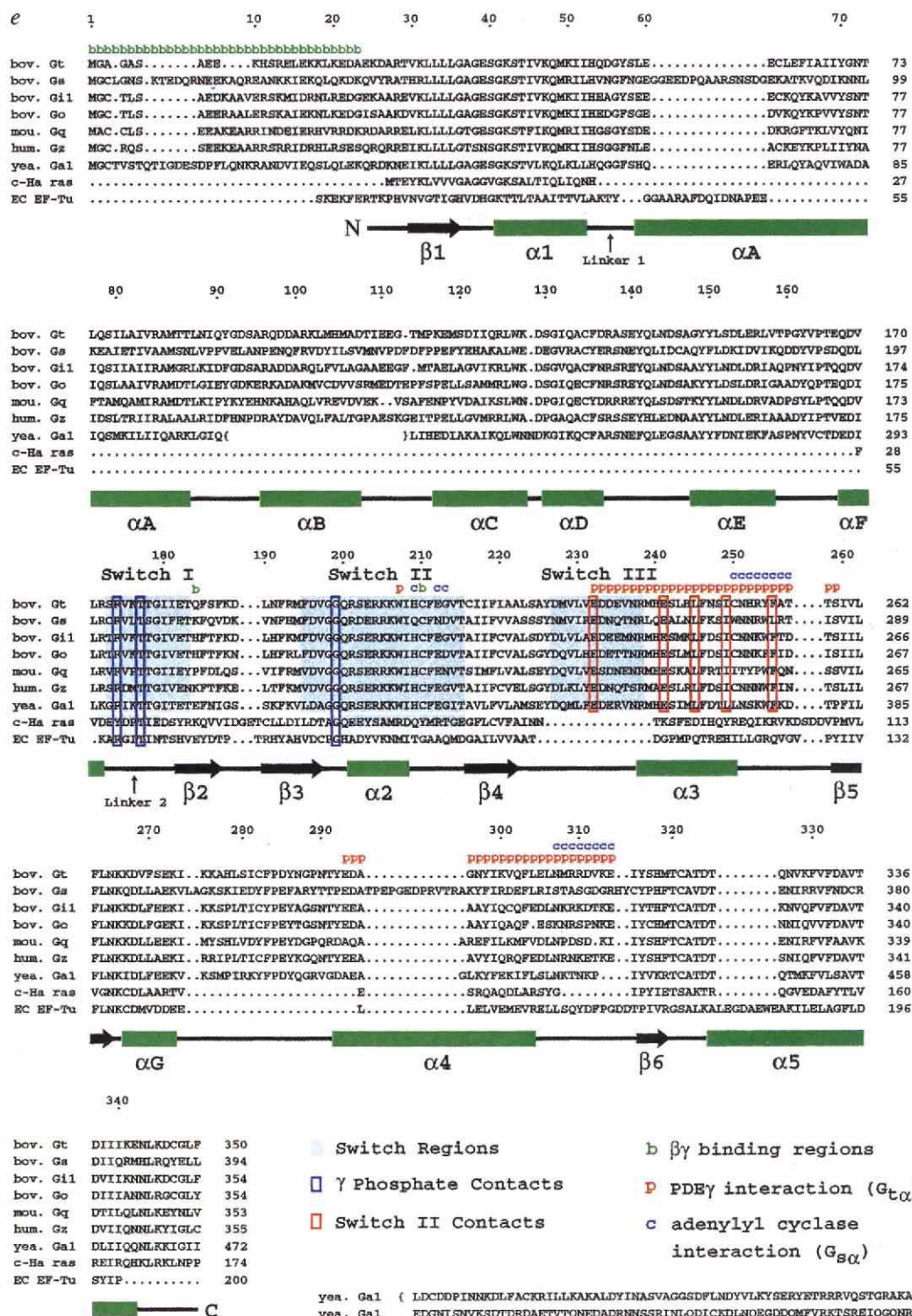


TABLE 1 Structure determination and refinement

Rotation search							Refined PC(Ω)		
Search structure	Θ_1	Euler angles		Θ_3	Highest peak	Highest False Peak			
GTPase domain	60.9	65.2		42.1	6.4 σ	3.8 σ			
Helical domain	59.4	62.9		47.8	6.9 σ	5.1 σ			
Both domains	60.0	65.8		43.2	6.8 σ	2.3 σ			
Translation search							Refined PC(Ω)		
Space group	x	Fractional Coordinates		z	Highest peak	Highest false peak			
I222	0.43	0.40		0.34	23 σ	15 σ			
I2 ₁ 2 ₁ 2 ₁	0.43	0.14		0.37	16 σ	16 σ			
G _{1a} ·GDP refinement									
Resolution limit (Å)	3.51	2.83	2.48	2.26	2.10	1.98	1.88	1.80	Total
Number of reflections	5,382	5,285	5,208	5,216	5,164	5,123	5,124	4,569	41,071
% Complete	100	100	100	99.9	99.7	99.1	98.8	88.9	98.3
$\langle I/\sigma \rangle$	31.4	26.6	19.7	14.7	10.7	7.60	4.85	3.42	19.0
R _{sym}	0.086	0.102	0.125	0.163	0.201	0.272	0.403	0.542	0.124
R-factor (all data)	0.198	0.178	0.198	0.207	0.217	0.234	0.283	0.331	0.211
R-factor (date>2.0 σ)	0.194	0.169	0.184	0.185	0.188	0.192	0.204	0.218	0.187
Free R-factor (all data)	0.231	0.203	0.219	0.222	0.236	0.267	0.320	0.350	0.236
Free R-factor (data>2.0 σ)	0.226	0.195	0.199	0.207	0.209	0.214	0.229	0.226	0.211
r.m.s. deviations	Bond lengths 0.010		Bond angles 1.46						
G _{1a} ·GTP- γ S refinement									
Resolution limit (Å)	3.51	2.83	2.48	2.26	2.10	1.98	1.88	1.80	Total
Number of reflections	13,384	13,193	12,874	12,407	12,093	11,768	11,506	11,367	98,592
% Complete	98.9	98.3	96.2	92.7	90.5	88.1	86.2	85.1	92.0
$\langle I/\sigma \rangle$	27.2	25.6	18.9	14.1	10.9	7.97	5.24	3.38	20.4
R _{sym}	0.064	0.067	0.082	0.104	0.132	0.172	0.243	0.337	0.077
R-factor (all data)	0.175	0.189	0.226	0.253	0.273	0.315	0.382	0.469	0.229
R-factor (date>2.0 σ)	0.175	0.187	0.218	0.239	0.25	0.271	0.290	0.301	0.211
Free R-factor (all data)	0.239	0.243	0.273	0.290	0.295	0.329	0.402	0.518	0.276
Free R-factor (data>2.0 σ)	0.239	0.241	0.266	0.276	0.278	0.289	0.299	0.319	0.258
r.m.s. deviations	Bond lengths 0.008 Å		Bond angles 1.31°						

G_{1a} β γ was isolated from photolysed bovine retinal rod outer segments (ROS) by selective extraction with GTP as described²⁷. G_{1a}·GDP was separated from G_{1a} β γ by chromatography on a cibacron blue column (HiTrap Blue, Pharmacia; 10 mM Tris, pH 7.5, 6 mM Mg₂SO₄, 1 mM EDTA, 10% glycerol, 0.1% β -mercaptoethanol, step to 0.15 M NaCl for 23 column volumes to elute G_{1a} β γ , and 0.15–1 M NaCl gradient over 10 column volumes to elute G_{1a}·GDP). A 325 amino-acid fragment (residues 26–350) of G_{1a}·GDP was prepared by endoproteinase LysC (Boehringer Mannheim) digestion of the full-length protein, reversibly activated with aluminium fluoride (50 μ M AlCl₃ and 15 mM NaF), for 12 h at 4 °C in 10 mM Tris, pH 7.5, 0.5 mM MgCl₂, 10% glycerol, and 0.1% β -mercaptoethanol. The proteolytic fragment was then purified on Mono-Q (10 mM Tris, pH 7.5, 0.5 mM MgCl₂, 50 μ M AlCl₃, 15 mM NaF, 10% glycerol, 0.1% β -mercaptoethanol, and 0.1–0.2 M NaCl gradient over 30 column volumes) in the presence of aluminium fluoride which was subsequently removed by ultrafiltration. Diffraction quality crystals of G_{1a}·GDP in the orthorhombic space group I222 were grown at 4 °C in microseeded hanging drops containing 13.3 mg ml⁻¹ protein, 6% PEG-8000, 6.67% glycerol, 33.3 mM MES, pH 6.0, 133 mM MgCl₂, and 0.1% β -mercaptoethanol equilibrated against a reservoir of 9% PEG-8000, 10% glycerol, 50 mM MES, pH 7.5, 200 mM MgCl₂, and 0.1% β -mercaptoethanol. Crystals appeared within 2 days and grew to maximum dimensions of 0.05 $\times$ 0.2 $\times$ 0.5 mm in 1–2 weeks. The unit cell dimensions are $a = 71.9$, $b = 87.8$, $c = 143.1$ Å. Crystals were stabilized in a cryosolution consisting of 20% PEG-8000, 30% glycerol, 200 mM MgCl₂, and 0.1% β -mercaptoethanol and flash frozen in liquid nitrogen-cooled liquid propane. Diffraction data to 1.8 Å for G_{1a}·GDP and G_{1a}·GTP- γ S were collected at the X-25 beamline of the National Synchrotron Light Source at Brookhaven National Laboratory, processed with DENZO (Z. Otwinowski), and scaled with SCALEPACK (Z. Otwinowski). Rotation searches with the GTPase and helical domains either separately or together gave similar solutions after Patterson correlation refinement²⁸, indicating that the two domains have the same relative orientation in both structures. A translation search using both domains yielded a clear solution in the space group I222 (but not I2₁2₁2₁) with an R-value of 45% after rigid body refinement. Inspection of the difference electron density maps ($2F_o - F_c$ and $F_o - F_c$) at this stage indicated several regions with poor density for main-chain atoms. These regions were removed from the initial model which was then refined in stages of increasing resolution (from 2.8 to 1.8 in steps of 0.2 Å) using a simulated annealing protocol with an initial temperature of 4,000 K. All computations for molecular replacement and subsequent refinement were done with X-PLOR 3.1²⁹. Interactive model building was done with the programs O³⁰ or Frodo³¹.

$$PC(\Omega) = \frac{\langle |E_{\text{obs}}|^2 |E_m(\Omega)|^2 - \langle |E_{\text{obs}}|^2 \rangle \langle |E_m(\Omega)|^2 \rangle \rangle}{[\langle |E_{\text{obs}}|^4 - \langle |E_{\text{obs}}|^2 \rangle^2 \rangle \langle |E_m(\Omega)|^4 - \langle |E_m(\Omega)|^2 \rangle^2 \rangle]^{1/2}}$$

where E_{obs} denotes the normalized observed structure factors and E_m denotes the normalized structure factors for the search model placed in a triclinic unit cell with geometry identical to that of the crystal²⁸.

$R_{\text{sym}} = \sum_h \langle |I_h - \bar{I}_h| \rangle / \sum_h \bar{I}_h$ where $\langle |I_h - \bar{I}_h| \rangle$ is the average of the absolute deviation of a reflection I_h from the average $\bar{I}_h$ of its symmetry and Friedel equivalents.

hydrogen-bonding and van der Waals contacts to the base, ribose, and α - as well as β -phosphates are nearly identical in both structures.

Activation of an α -subunit

As shown in Fig. 1, the structures of G_{1a}·GDP and G_{1a}·GTP- γ S are quite similar. In fact, if the switch regions described below are excluded, the r.m.s. deviation after superposition of the remaining 281 C α atoms is only 0.8 Å. Thus, to a good approximation, the structural differences induced by nucleotide exchange are localized to three adjacent regions on one face of the protein (Fig. 1). Subsequently, we will refer to these regions as switch I, II and III. Switch I (Ser 173–Thr 183) encompasses linker 2 and extends partially into the β 2 strand. Switch II

(Phe 195–Thr 215) begins near the C terminus of β 3, extends through α 2, and includes the α 2– β 4 loop. Switch III (Asp 227–Arg 238) spans the β 4– α 3 loop and occurs within a sequence segment (I2) that is the second of four sequence inserts (I1–I4) with respect to the Ras sequence. The switch I and II regions have counterparts in Ras^{8,10} and EF-Tu^{11,12,44} by virtue of similar contacts between the γ -phosphate of GTP and two highly conserved residues, Thr 177 in switch I and Gly 199 in switch II. Switch III, by contrast, is unique to heterotrimeric G proteins and the conformational changes in this region (described below) appear to occur as a secondary response propagated from switch II through a network of interactions, primarily ionic, that are conserved in heterotrimeric G proteins. With the exception of a short stretch of residues (Gln 143–Asn 145) which in G_{1a}·GTP-

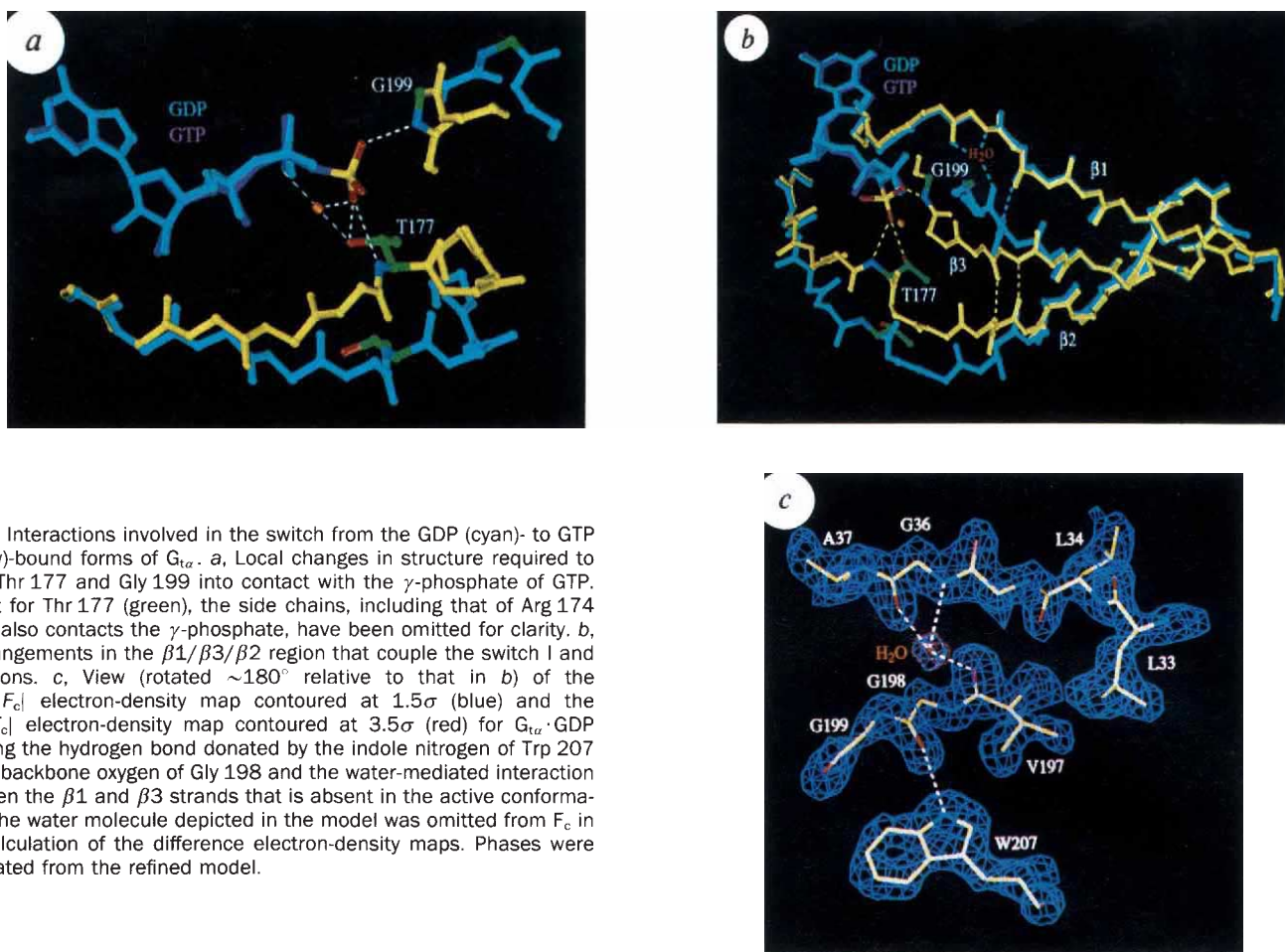


FIG. 2 Interactions involved in the switch from the GDP (cyan)- to GTP (yellow)-bound forms of $G_{i\alpha}$. **a**, Local changes in structure required to bring Thr 177 and Gly 199 into contact with the γ -phosphate of GTP. Except for Thr 177 (green), the side chains, including that of Arg 174 which also contacts the γ -phosphate, have been omitted for clarity. **b**, Rearrangements in the $\beta 1/\beta 3/\beta 2$ region that couple the switch I and II regions. **c**, View (rotated $\sim 180^\circ$ relative to that in **b**) of the $2[F_o] - |F_c|$ electron-density map contoured at 1.5σ (blue) and the $|F_o| - |F_c|$ electron-density map contoured at 3.5σ (red) for $G_{i\alpha} \cdot \text{GDP}$ showing the hydrogen bond donated by the indole nitrogen of Trp 207 to the backbone oxygen of Gly 198 and the water-mediated interaction between the $\beta 1$ and $\beta 3$ strands that is absent in the active conformation. The water molecule depicted in the model was omitted from F_c in the calculation of the difference electron-density maps. Phases were calculated from the refined model.

γS lie in contact with the switch III region, both the conformation of the helical domain and its orientation with respect to the GTPase domain are essentially unaffected by nucleotide exchange. Likewise, the I3 and I4 regions are also unchanged.

Structural changes in the switch I region are induced by hydrogen bonds between the γ -phosphate of GTP and Thr 177 (Fig. 2a) and to a lesser degree the cholera toxin-sensitive residue Arg 174. Specifically, the O3 oxygen of the γ -phosphate accepts hydrogen bonds from both the main-chain N and side-chain OH of Thr 177, the oxygen of which is also coordinated to the Mg^{2+} ion, whereas the γ -S interacts with the guanidyl group of Arg 174. The structural changes occurring in the switch I region are conceptually simple. After nucleotide exchange, linker 2 is drawn essentially as a whole towards the γ -phosphate, bringing the side chain oxygen of Thr 177 into the coordination sphere of the Mg^{2+} ion where it replaces one of the water ligands observed in the structure of the GDP form. The average main chain B-factor for residues in the switch I region is a factor of 2 lower in the $G_{i\alpha} \cdot \text{GTP} \cdot \gamma S$ structure, suggesting the importance of the stabilizing contacts with the γ -phosphate and Mg^{2+} ion to the active conformation.

Changes in the switch II region are initiated by a hydrogen bond between the peptide NH of Gly 199 and the γ -phosphate of GTP (Fig. 2a). Gly 199 is located in a short loop preceding $\alpha 2$ which in $G_{i\alpha} \cdot \text{GDP}$ forms a severely distorted 3–10 helix that terminates in a type I reverse turn stabilized by a salt bridge between His 209 and Glu 212. Movements in this loop are coupled to structural changes in $\alpha 2$ through a hydrogen bond from the indole NH of Trp 207 to the backbone carbonyl of Gly 198 (Fig. 2c). To bring Gly 199 into contact with the γ -phosphate of GTP, the switch II region is both stretched and rotated (roughly a quarter turn anticlockwise when viewed from the phosphate) relative to its conformation in $G_{i\alpha} \cdot \text{GDP}$. The

net effects are to: (1) straighten $\alpha 2$ into a more ideal 3–10 helix (Fig. 1c, d); (2) disrupt the reverse turn at the end of $\alpha 2$ and the associated interaction between His 209 and Glu 212 (Fig. 3c, d); (3) bring the exposed side chains of the conserved residues Arg 201, Arg 204 and Trp 207 into contact with conserved residues in $\alpha 3$ that are unique to heterotrimeric G proteins (Fig. 3a, b). In $G_{i\alpha} \cdot \text{GTP} \cdot \gamma S$, the guanadinium groups of Arg 201 and Arg 204 ion pair with the carboxylate group of Glu 241, whereas Trp 207 is buried between the side chains of Leu 245 and Ile 249. A further consequence of the conformational twist in the switch II region is that the end of $\beta 3$ (from Phe 195 to Gly 199) pulls away from $\beta 1$, disrupting one direct and one water-mediated hydrogen bond (Fig. 2b). These disrupted interactions are replaced by two new hydrogen bonds between $\beta 3$ (the strand preceding Gly 199 in switch II) and $\beta 2$ (the strand following linker 2 in switch I). The new hydrogen bonds couple the structural changes in the switch I and II regions, thereby lending cooperativity to the switch mechanism. Contrary to our initial expectations¹³, the structural changes in the switch II region do not propagate to $\alpha 3$. Instead, it appears that $\alpha 3$ serves as a rigid scaffold for residues involved in stabilizing the active conformation of the switch II region.

The changes in the switch II region explain a variety of observations regarding the conformation of this region in solution. For example, proteolysis studies show that $G_{i\alpha} \cdot \text{GDP}$ is sensitive to cleavage by trypsin¹⁶ at Arg 204 and chymotrypsin¹⁷ at Trp 207, whereas both sites are protected against proteolysis in the GTP conformation. Likewise, the amplitude of tryptophan fluorescence for $G_{i\alpha} \cdot \text{GTP} \cdot \gamma S$ is enhanced relative to that for $G_{i\alpha} \cdot \text{GDP}$ consistent with one of transducin- α 's two tryptophans having a more polar environment in the GDP conformation¹⁸. Mutation of Trp 207 to Phe eliminates the fluorescence change associated with activation¹⁹. Both results would be expected

from the switch II structural changes which rotate the Arg 204 and Trp 207 side chains from solvent-exposed conformations into ordered interactions with residues from $\alpha 3$ and switch III.

Switch III responds to switch II by forming an elaborate interdependent network of polar interactions (Fig. 3c, d) with functional groups that have been positioned and firmly buttressed by the GTP-induced rearrangements in the switch II region. The carboxylate group of the conserved Glu 232 in switch III plays a crucial role by forming a direct interaction with the backbone amide of Arg 201 as well as water-mediated interactions with the guanidino NH of Arg 204 and the carbonyl oxygen of Gly 199 (Fig. 3d).

All of the critical residues for the conformational switch in $G_{1\alpha}$ are also present in the other heterotrimeric G-protein α -subunits. Consequently, we anticipate that the same basic switch mechanism, including the coupling between the switch I and II regions and the propagation of the switch II changes to the switch III region, will hold for all members of the heterotrimeric G-protein family.

Implications for 'release' of activated G_{α}

A characteristic consequence of nucleotide exchange in heterotrimeric G proteins is reduced affinity of activated G_{α} for $G_{\beta\gamma}$.

Extensive studies of the Gly 226 to Ala mutation in G_{sa} (Gly 199 in $G_{1\alpha}$), first identified as the H21A clone in S49 cells²⁰ and later introduced as a site-specific mutation²¹, demonstrate that release of $G_{\beta\gamma}$ is a necessary event accompanying G_{α} activation and that the conformational changes in switch II are important for regulating affinity for $G_{\beta\gamma}$. In neither conformation does Gly 199 violate the $\phi\psi$ angles permitted by residues with $c\beta$ substituents. However, a $c\beta$ substituent cannot be tolerated at this position because it would result in an unfavourable steric interaction with the carbonyl oxygen of Gly 36 in the GTP but not in the GDP conformation. Figure 4 shows that the GDP-bound form has a cavity, formed by switch II, $\alpha 3$ and switch III residues, that closes in response to the GTP-induced changes described above. The polar and non-polar residues in $\alpha 2$ protrude as an open flap in the GDP-bound form and present a potential binding site for $G_{\beta\gamma}$ that would be lost on nucleotide exchange. One region of G_{α} important for binding to $G_{\beta\gamma}$ is the N terminus (see ref. 3) which is missing in our proteolysed form of $G_{1\alpha}$. A region adjacent to $\alpha 2$ has been implicated in $G_{\beta\gamma}$ binding by a second site revertant at position 307 in the yeast mating factor G_{α} (corresponding to Gln 184 in $G_{1\alpha}$) which restores affinity for $G_{\beta\gamma}$ mutants that otherwise fail to interact properly with G_{α} ^{22,23}. Moreover, a cysteine in the switch II

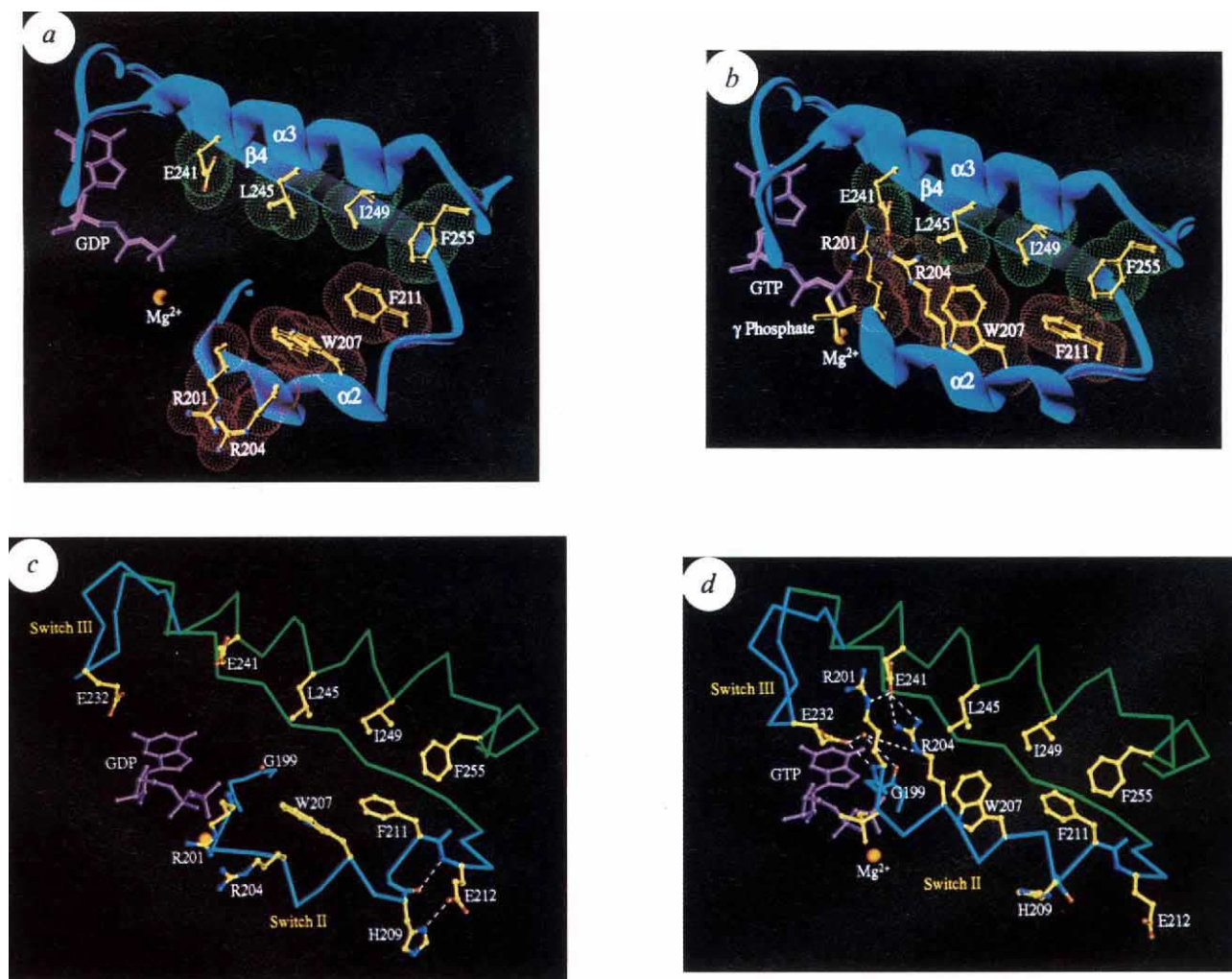


FIG. 3 Conformational changes in the switch II and III regions showing how the side chains of residues in $\alpha 2$ rotate from solvent-exposed orientations in the inactive (GDP-bound) conformation to form an extensive interface with residues from the $\beta 4$ - $\alpha 3$ loop and $\alpha 3$ in the active (GTP- γ S-bound) conformation. a, Ribbon drawing of the $\alpha 2$ - $\alpha 3$ region in $G_{1\alpha}$ ·GDP depicting the solvent-exposed side chains of the highly conserved residues Arg 201, Arg 204, Trp 207 and Phe 211 in $\alpha 2$ and Glu 241, Leu 245, Ile 249 and Phe 255 in $\alpha 3$. The GDP is coloured

purple and the Mg^{2+} ion appears as an orange sphere. b, Ribbon drawing of the $\alpha 2$ - $\alpha 3$ region in $G_{1\alpha}$ ·GTP- γ S showing the extensive interface formed between the side chains of residues in $\alpha 2$ and $\alpha 3$. c, Skeletal model of the $\alpha 2$ - $\alpha 3$ region showing α carbons and selected side chains in $G_{1\alpha}$ ·GDP. d, Same view as c showing the interactions between the Glu 232 carboxylate and the backbone NH of Arg 201 (direct hydrogen bond) and the carbonyl of Gly 199 (water mediated) that couple switch III to switch II.

region of G_{α} (Cys 210 in $G_{1\alpha}$) can be chemically crosslinked to bound $G_{\beta\gamma}$ ²⁴. In the context of the structural changes, these studies suggest that the switch II and perhaps the switch I region may play a role in modulating affinity for the $\beta\gamma$ subunits. The concomitant decrease in affinity for receptor could result from either a direct interaction of the receptor with one of the switch regions of G_{α} or, alternatively, as a consequence of the decreased affinity for $G_{\beta\gamma}$.

Implications for effector activation

At least one of the regions involved in effector interaction must undergo conformational changes on nucleotide exchange. For two well characterized G_{α} -effector interactions, $G_{1\alpha}$ ·GTP activation of cGMP phosphodiesterase (cGMP PDE) and G_{sa} ·GTP activation of adenylyl cyclase, effector activation regions have been inferred from biochemical²⁵ and mutational data^{19,26}. Together these studies define a contiguous surface consisting of residues from $\alpha 2$, $\alpha 3$, the latter part of $\alpha 4$, and the four loops $\alpha 2$ - $\beta 4$, $\alpha 3$ - $\beta 5$, $\alpha 4$ - $\beta 6$, and $\beta 4$ - $\alpha 3$ (see Fig. 4 of ref. 13). Of these, two occur within switch regions; the $\alpha 2$ - $\beta 4$ loop lies at the end of switch II and the $\beta 4$ - $\alpha 3$ loop corresponds to the switch III region.

$G_{1\alpha}$ ·GTP activates cGMP PDE by binding to and displacing its inhibitory γ -subunits⁴. Studies with synthetic peptides have implicated two segments of $G_{1\alpha}$ in binding to PDE γ . A peptide, encompassing $\alpha 4$ and the $\alpha 4$ - $\beta 6$ loop, binds PDE γ (dissociation constant, $K_d \sim 2 \mu\text{M}$) and activates PDE²⁵. However, we do not observe significant conformational changes in the $\alpha 4$ - $\beta 6$ activating region (293-314). In fact, the $\alpha 4$ - $\beta 6$ loop appears to be quite flexible in both nucleotide-bound forms of $G_{1\alpha}$, as shown by equally large main chain B-factors ($\langle B \rangle \sim 50$). A second peptide, beginning in the switch III region and extending through $\alpha 3$, binds PDE γ but does not activate PDE (J. Mills, manuscript in preparation). Switch III contains a cluster of acidic residues Glu 232-Asp-Asp-Glu 235 that could potentially interact with the highly basic central segment of PDE γ . Mutational studies

have also implicated a switch II residue, Trp 207, in the interaction with PDE γ ¹⁹.

In vivo studies with $G_{1\alpha}$ - G_{sa} chimaeras have implicated three loops, $\alpha 2$ - $\beta 4$, $\alpha 3$ - $\beta 5$, and $\alpha 4$ - $\beta 6$, in G_{sa} activation of adenylyl cyclase²⁶. Of these only the $\alpha 2$ - $\beta 4$ loop exhibits a clearly identifiable structural change on nucleotide exchange. Indeed the sequence segment of $G_{1\alpha}$ that was inserted in the $\alpha 2$ - $\beta 4$ loop is identical to that of $G_{1\alpha}$ and maps directly onto the reverse turn at the end of $\alpha 2$ (see Fig. 1e), suggesting a plausible mechanism for G_{sa} activation of adenylyl cyclase in which disruption of this reverse turn on nucleotide exchange releases residues that, with the other effector interaction regions of G_{sa} , cooperatively enhance affinity for adenylyl cyclase.

Comparison with p21 Ras and EF-Tu

The switch I and II regions of $G_{1\alpha}$ are analogous to switch regions in Ras⁸⁻¹⁰ and EF-Tu^{11,12,44} whereas switch III is unique to the heterotrimeric family of G proteins. Aside from the conserved threonine and glycine residues, the switch I and II regions exhibit little if any sequence similarity to the corresponding switch regions of Ras or EF-Tu and, despite highly conserved contacts with the γ -phosphate of GTP, both the nature and extent of the resulting structural changes are quite distinct. In $G_{1\alpha}$, the changes in the switch I region begin at Ser 173, extend through linker 2, and include a portion of $\beta 3$. Nucleotide exchange is accompanied by movement of the whole of this region. In Ras, by contrast, the key element of the switch I conformational rearrangement is a 180° flip of the peptide backbone bringing Thr 35 (corresponding to Thr 177 in $G_{1\alpha}$) from a solvent-exposed orientation in the GDP form into contact with the γ -phosphate and Mg^{2+} ion. The changes in the corresponding region of EF-Tu are obscured by the absence, due to proteolysis, of 17 residues N-terminal to the conserved threonine in the EF-Tu·GDP structure¹¹.

Likewise, the changes in the switch II region are also distinctly different. Although a portion of the switch II region is disordered

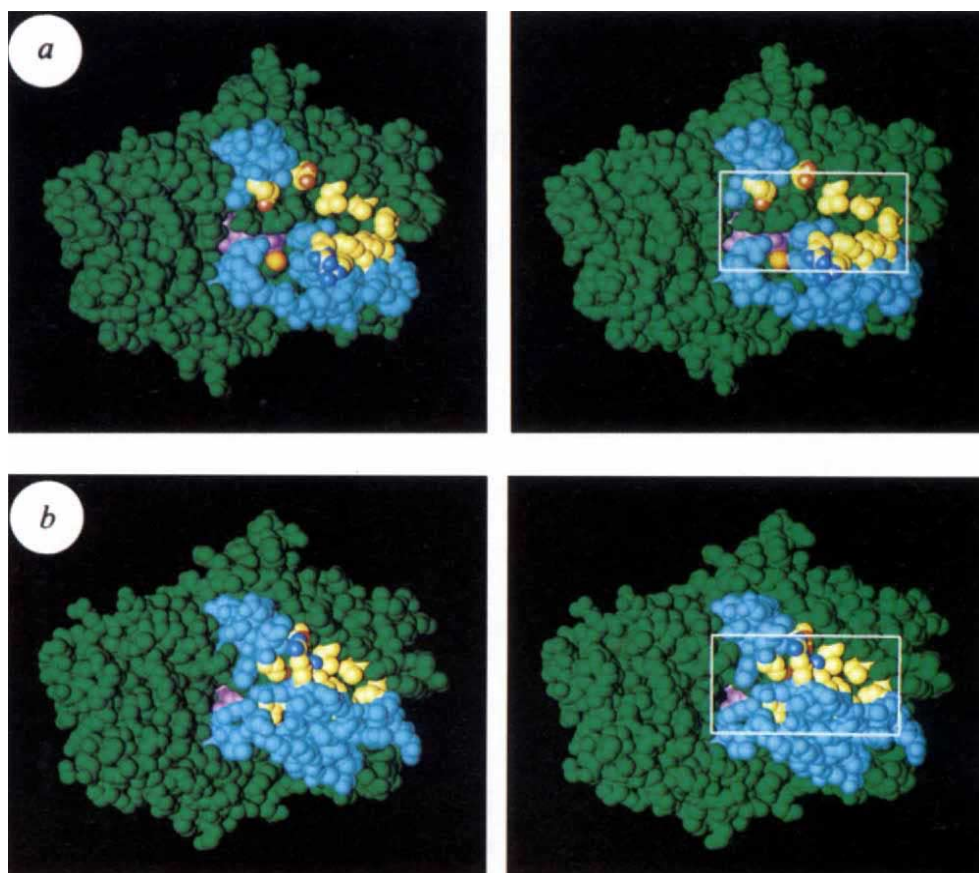


FIG. 4 Stereo pair of space filling models showing the same structures and view as in Fig. 1b and c. Residues in the switch regions are shown in cyan except for the gold residues which are those that propagate/stabilize the structural transitions induced by the γ -phosphate. The box outlines a cavity in $G_{1\alpha}$ ·GDP that closes on introduction of GTP and may be a region of $G_{1\alpha}$ that along with the amino and carboxy termini modulates the affinity of $G_{1\alpha}$ for $G_{\beta\gamma}$ and receptor. a, $G_{1\alpha}$ ·GDP. b, $G_{1\alpha}$ ·GTP· γ S.

in the GDP form of Ras, the conformational changes in the switch II region appear to resemble the changes in EF-Tu where GTP binding results in the addition of an extra helical turn at the carboxyl end of $\alpha 2$ at the expense of a turn at the beginning of the helix¹². In conjunction with this, the $\alpha 2$ helix rotates by $\sim 40^\circ$ about an axis perpendicular to the helical axis. In $G_{1\alpha}$, rather than losing a turn at the beginning and gaining a turn at the end of $\alpha 2$, the GTP-induced changes straighten out an otherwise distorted 3_{10} helix while disrupting a reverse turn at the end of the helix. Furthermore, the rotation of the helical axis seen in p21 Ras and EF-Tu is replaced by a $\sim 90^\circ$ rotation roughly parallel to (but not coincident with) the helical axis. Finally, the

partial unzipping of $\beta 3$ - $\beta 1$ and concomitant extension of $\beta 3$ - $\beta 2$ that couples the switch I and II regions in $G_{1\alpha}$, does not occur in either Ras⁸⁻¹⁰ or EF-Tu¹². Most distinctive is the presence in $G_{1\alpha}$ of a set of polar and nonpolar linkages that couple the primary structural changes, induced by interaction with the γ -phosphate, to nearby surface elements, some of which have been implicated in interactions with other components of the signalling system. Because these linkages are formed by residues that are unique to and conserved in $G_{1\alpha}$ subunits we suggest that they provide the basis for a common switch mechanism in heterotrimeric G-protein-coupled signal transduction. $\square$

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LETTERS TO NATURE

Dust depletion in the inner disk of β Pictoris as a possible indicator of planets

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It is not yet possible to see planets orbiting other stars, although this may soon change as observing methods improve¹. Indirect evidence for the presence of circumstellar dust disks out of which planets could form has been obtained for several stars, in the form of excess infrared emission, presumed to be from the hot dust^{2,3}. Planets orbiting in such dust disks would be expected to sweep out dust-free tracks⁴. Indirect evidence for dust-free regions has been reported⁵⁻⁹, based on an analysis of the spectral energy distribution, but the interpretation of the observation is not unique^{7,9}. Here we present an infrared image of the inner dust disk of the star β Pictoris with a linear resolution of 5 astronomical units (AU), equivalent to the distance from the Sun to Jupiter. We find that the dust is asymmetrically distributed and is clearly depleted within 40 AU of the star, which we interpret as indicating the possible presence of at least one planetary body orbiting β Pictoris.

The 10- μ m region is one of the best spectral ranges to achieve high angular resolution for an object like the β Pictoris disk.

The outer part of the disk (>100 AU) has been first imaged with ground-based observations in the visible part of the spectrum¹⁰. But because of too high a contrast between the star and the disk emission, the disk structure within 2.5 arcsec of the star (40 AU at the distance of β Pictoris, 16 pc) has been inaccessible until now, even with sophisticated techniques such as coronagraphic optics or anti-blooming CCDs (charge-coupled devices)^{11,12}. Observing at 10 μ m alleviates this problem, because the thermal emission from dust is comparable in intensity to the photospheric emission of the star, while the diffraction-limited angular resolution is still high: 0.7 arcsec for a 3-m telescope. This resolution, which has not been achieved with either single detectors or with arrays of discrete detectors^{8,9} is now accessible with the new generation of integrated detector arrays. The European Southern Observatory (ESO) has recently acquired an instrument that employs such a device: the TIMMI camera, built by the Service d'Astrophysique at Saclay¹³. The detector is a monolithic 64×64 gallium-doped silicon array hybridized by indium bumps to a direct read out circuit, optimized by the LIR (Laboratoire Infra-Rouge) at the Grenoble Centre d'Etudes Nucléaires for the high-background conditions of ground-based broad-band imaging. The TIMMI camera mounted on the 3.6-m telescope at the ESO, La Silla, Chile was used during five nights (6-11 January 1993) for imaging the β Pictoris dust disk at sub-arcsec resolution.

The disk structure observed after an on-source integration time of 75 min (spread over three nights) with a pixel field of view of 0.3 arcsec and a 10.5-13.3 μ m band-pass filter is shown in Figs 1 (main figure) and 2; the signal-to-noise ratio at the

peak is ~ 100 . The weather was good during the observations, and the air-mass was always below 1.3. The photometry was done using the nearby (5°) star α Carinae; a total (star + disk) flux of 2.28 Jy was found within an equivalent beam diameter of 4 arcsec (before deconvolution), in agreement with previous measurements^{14,15}. The star α Carinae was also used to determine the point-spread function (the smearing of light by the atmosphere and the instruments) and to subtract the β Pictoris stellar contribution (the scaling factor between the two stars was taken to be 0.0111, ref. 14). The disk contribution is large (always $>40\%$ of the total flux), so that removing the stellar contribution is not critical; the uncertainty in the removed part is 20% in each pixel (see legend of Fig. 2), whereas the central disk contribution would be hidden only for uncertainties greater than 60%. To go beyond the angular resolution limitation imposed by the point-spread function (full-width half-maximum of ~ 0.9 arcsec), we used the Richardson-Lucy deconvolution technique^{16,17} combined with wavelet filtering¹⁸, which prevents noise amplification. We monitored the point-spread function by interleaving the observations of β Pictoris with observations of the two nearby ESO catalogue reference stars, α Carinae and γ Reticuli. The latter star was used to determine the residuals of the deconvolution method (see Fig. 1, inset).

Fig. 1 confirms without ambiguity the previous claims that the β Pictoris dust disk is extended at $10\ \mu\text{m}$ (refs 8, 9); the emission extends to more than 4 arcsec from the star. The second striking feature directly seen in the image is the asymmetric morphology of the disk. Neither property is an artefact of the data

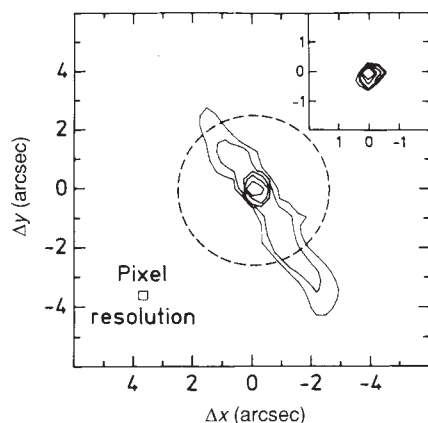


FIG. 1 Main figure, the deconvolved β Pictoris dust disk, (the star contribution has been removed), as seen at $11.9\ \mu\text{m}$ with a spatial resolution of 0.3 arcsec (5 AU at the distance of the object). The highest contour corresponds to $1.2\ \text{Jy arcsec}^{-2}$, at each subsequent contour the flux is divided by 3. North is top; east is left. The usual chopping and nodding technique was used to remove the atmosphere and telescope background contribution. The secondary chopping mirror unit of 3.6 m ESO telescope was used at a frequency of 5 Hz; changes of telescope position (nodding) was done every 40 s. The total on-source integration time (half of the total time) was 75 min. The region inside the dashed circle (2.5-arcsec radius) was inaccessible to previous observations in visible radiation. A broad-band filter ($10.5\text{--}13.3\ \mu\text{m}$) was used. The large well depth of the detectors ($\sim 3 \times 10^7$ electrons per pixel) allows the use of broad-band filters without saturation by the huge photon background emitted at $10\ \mu\text{m}$ by the telescope and the atmosphere. These detectors are a by-product of the detector development programme undertaken for ISOCAM, an imaging instrument to be installed on the Infrared Space Observatory (ISO)²⁵. Inset, Result of deconvolution of two reference stars, α Carinae and γ Reticuli, showing that the residuals after deconvolution are at a low level; (The flux is divided by 3 between adjacent contours, as on the main Figure). These residuals are conservative because γ Reticuli is much further away from α Carinae than β Pictoris (30° instead of 5°), so that aberrations dependent on the telescope position are included in the residuals.

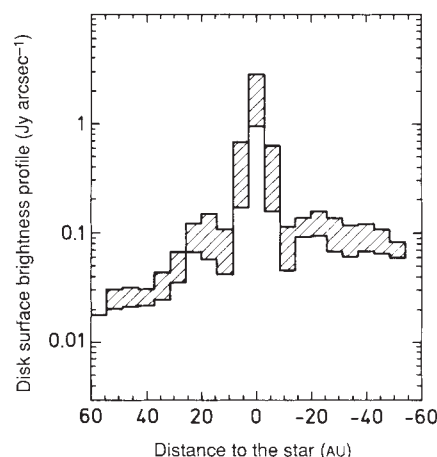


FIG. 2 Surface brightness profile of the disk integrated over 3 pixels perpendicular to the disk plane (in order to avoid sampling errors due to the fact that the disk is not aligned with the array; the flux outside these 3 pixels is negligible), as a function of the distance to β Pictoris (east is positive) in astronomical units (1 arcsec = 16 AU). Half of the flux is in the central bin, 70% is in the three central bins. The shaded region corresponds to conservative uncertainties in the process of star-contribution removal and deconvolution. The photometric error over a beam with a diameter of 9 pixels and extending over the two-dimensional image was measured to be $\pm 7\%$ (peak-to-peak variation for 10 measurements). If we also consider sampling errors, we obtain $\pm 15\%$ on a pixel (peak-to-peak variation of the central pixel for 10 measurements). Finally, we have taken an uncertainty of $\pm 20\%$ in the β Pictoris stellar contribution, which includes an additional uncertainty of 5% on the scaling factor between α Carinae and β Pictoris. In reality these uncertainties are too conservative. As the observations of α Carinae have been interspersed with the observations of β Pictoris, we were able to monitor the time variations in the photometry and point spread function (PSF). The sampling error has also been overestimated, because co-adding several observations of the same object obtained at various positions on the array averages the sampling error, and decreases the difference between the sampling of the object and of the reference star. We have also overestimated the uncertainties of the PSF variation with the telescope position, by adding linearly the uncertainties obtained when using γ Reticuli (30° away from β Pictoris) instead of α Carinae (5° away from β Pictoris).

analysis, as each is already visible in the raw data. To obtain the dust density profile, some modelling is necessary, because the observed surface brightness also depends on the grain temperature T , on the grain absorption coefficient Q_{abs} and on the grain size distribution. Furthermore, as the disk is seen edge-on¹⁰ and asymmetric, we can only derive directly from the observations the mean surface density of the face-on disk. An estimate of the dust absorption coefficient, size distribution and temperature can be made, because we know the heat source (an A star at 8,200 K and of 6.5 solar luminosity) and the nature of some of the particles (silicate grains, as shown by the detection in a 5 arcsec beam of the silicate feature near $10\ \mu\text{m}$ ^{14,15,19}). The derived profile of the mean surface density is shown in Fig. 3 (see Fig. legend for details of the calculations). We conclude that there is no strict void of matter near the star, but rather a relatively steep decrease of the mean density when approaching it.

The density distribution inferred for the inner region is quite different from that in the outer region ($>100\ \text{AU}$) deduced from visible observations^{7,10}. The beginning of a transition region has been observed at $\sim 100\ \text{AU}$ by recent visible observations which can distinguish structures farther than $40\ \text{AU}$ from the star^{11,12}, which is further than the size of Pluto's orbit. This transition is attributed to the sublimation of ice particles, as supported by the

two-component models of ref. 9. Given the strong dependence of the sublimation timescale on the dust temperature²⁰, the transition region should not be very large (a few tens of AU at most), so that we do not expect this mechanism to be able to reproduce the density decrease observed within 30 AU, except if the dust particles had a high eccentricity. On the other hand, planets are able to produce a steep deficiency of matter in this region; the matter can be either swept up by the planet¹⁰, or trapped in gravitational resonances induced by the planet (for example, ref. 21). Given the age of the β Pictoris system ($(1-2) \times 10^8$ yr), planets could have formed up to a maximum radius of ~ 20 AU (the size of Uranus' orbit), according to Nakano²², but the models of planet formation are still uncertain. Detailed calculations of the effect of planets on a dust disk, taking into account gravitation and Poynting–Robertson effects but neglecting particle collisions, have been presented recently²¹. These models show that planets with masses as low as a few Earth masses are able to produce a large depletion of matter inside their orbit on a timescale of 10^5 – 10^6 yr. The density predicted by these models agrees with the observations in the 10–30 AU region, but the predicted profile is too steep inside 10 AU (Fig. 3). Particle collisions may help in filling the innermost region.

Another possibility is the direct injection of particles into the innermost region (<3 AU) by comet evaporation, although this was introduced in a completely different context: the interpretation of the redshifted and variable gaseous absorption lines observed in the visible and ultraviolet radiation²³. A total of 1,000 comets per year, each a kilometre in size, is required to explain the observations²⁴; these would give rise to a dust inflow rate of $\sim 5 \times 10^{18}$ g yr⁻¹. Evidence that the inner 10- μ m emission could originate from cometary materials is provided by the shape of the silicate feature observed at 10 μ m (refs 13, 14, 19). The total dust-mass necessary to account for the 10- μ m observations of the innermost region presented here is $\sim 6 \times 10^{20}$ g. The associated dust flow varies between 6×10^{17} g yr⁻¹ to 6×10^{20} g yr⁻¹ according to the dust removal process under consideration; (radiation pressure which rapidly blows out small particles or Poynting–Robertson effect which makes the particles slowly fall onto the star). Comets therefore are possible candidates as sources of replenishment of the innermost region observed here.

If a planet is present near β Pictoris, its orbit is probably eccentric, as are most of the planetary orbits in our Solar System. A planet with even a moderate eccentricity of 0.02 should generate a spectacular asymmetry, such as arc-like structures²¹ in the dust disk. In this context, the asymmetry observed here can be considered as more evidence in favour of the presence of a planet near β Pictoris. Such an asymmetry should vary in time, and it would be interesting to monitor. Some patience may be required, however, because the half-orbital period of an object 20 AU from the central star is 36 years. $\square$

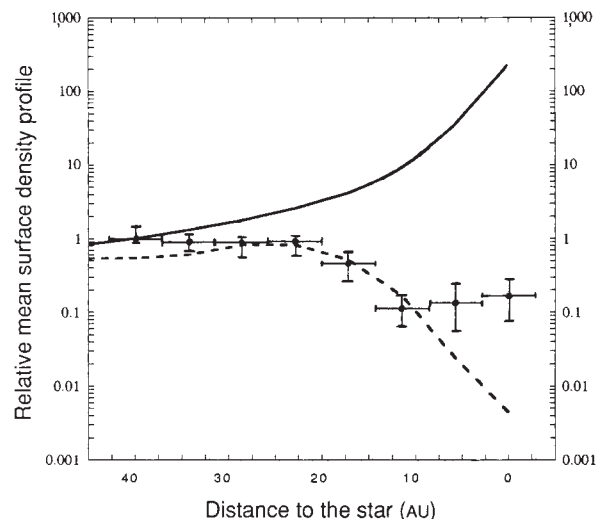


FIG. 3 Profile (crosses) of the face-on surface-density averaged along the line of sight, l , responsible for the observed flux of Fig. 2, S_{obs} , at an angular distance θ from the star in the disk direction perpendicular to the line of sight. (We do not consider a volume density as we have integrated the observed flux over the direction perpendicular to the disk plane.) $B_{\lambda}(T)$ is the blackbody brightness, $N(a)da$ is the probability of having particles with sizes between a and $a+da$.

$$\langle n \rangle_{\text{obs}}(\theta) = S_{\text{obs}}(\theta) / \left[\int_{-\infty}^{\infty} \int_{\text{size dist.}} \int_{\text{filter}} \pi a^2 Q_{\text{abs}}(a) B_{\lambda}(T(r, a)) N(a) (D^{-2}) dl da d\lambda \right] \quad (1)$$

(the radial coordinate r is related to θ and l via the usual relation $r = \sqrt{l^2 + \theta^2} \times D$, where D is the β Pictoris–earth distance). We have assumed silicate grains with an absorption coefficient Q_{abs} taken from ref. 26 and a size distribution as in¹⁹ ($N(a) = 9.8a^{-2} \mu\text{m}^{-1}$ with a cut-off and a cut-on, respectively, at 0.1 μm and 5 μm) and independent of the distance to the star r . Such a distribution (flatter than usual) has also been found in another main-sequence star with an infrared excess, HD98800 (ref. 27). The particle-size dependence of the radiation pressure (small particles are rapidly blown out of the system) may explain the flatness of the distribution. The profile has been limited to 40 AU, because at greater distances the uncertainties due to the modelling entering in equation (1) become significant, given that the calculated temperature (<100 K) is far from 300 K, the temperature for which the emission peaks at 10 μm . Note that even if the observed brightness keeps on increasing towards the star (Fig. 2), the density profile must decrease to compensate for the strong increase in the dust temperature near the star. The density in the central part is 3×10^{18} particles m⁻². The observations are compared with two models. The solid line represents the profile expected for a radial density following a $r^{-1.7}$ law (outer-region density integrated upon the disk thickness). The dotted line represents the dust profile expected if a planet was at 20 AU according to the model calculations of ref. 21.

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High-power laser diodes based on InGaAsP alloys

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HIGH-POWER, high-coherence solid-state lasers, based on dielectric materials such as ruby or Nd:YAG (yttrium aluminium garnet), have many civilian and military applications. The active media in these lasers are insulating, and must therefore be excited (or 'pumped') by optical, rather than electrical, means. Conventional gas-discharge lamps can be used as the pumping source, but semiconductor diode lasers are more efficient, as their wavelength can be tailored to match the absorption properties of the lasing material. Semiconducting AlGaAs alloys are widely used for this purpose^{1,2}, but oxidation of the aluminium and the spreading of defects during device operation limit the lifetime of the diodes³, and hence the reliability of the system as a whole. Aluminium-free InGaAsP compounds, on the other hand, do not have these lifetime-limiting properties⁴⁻⁸. We report here the fabrication of high-power lasers based on InGaAsP (lattice-matched to GaAs substrates), which operate over the same wavelength range as conventional AlGaAs laser diodes and show significantly improved reliability. The other optical and electrical properties of these diodes are either comparable or superior to those of the AlGaAs system.

Semiconductor p-n junction lasers (laser diodes) demonstrate high conversion efficiency of electrical to optical power (>50%), but the quality of the beam (particularly the coherence) is not as good as solid-state lasers. Pumping solid-state lasers with laser diodes therefore makes it possible to combine the advantages of both kinds of lasers. To optimize the performance of the system, the wavelength of the pumping laser diodes has to fit the absorption spectrum of solid-state (pumped) materials.

Up to now, commercially available diode-pumped Nd:YAG solid-state lasers used diode lasers emitting at 0.808 μm , and fabricated from materials in the $\text{Al}_{1-x}\text{Ga}_x\text{As}$ system. However, the interaction of Al with oxygen leads to oxide formation at the mirror facet, and enhances the non-radiative recombination of injected carriers nearby. This leads to overheating of the mirror, and decreases the lifetime of the device. Another lifetime-limiting factor is the formation of dark line defects, as a consequence of the spreading of dislocations in the active region during high-power operation³.

Both of these problems can be circumvented by replacing AlGaAs with the Al-free $\text{In}_{1-x}\text{Ga}_x\text{As}_y\text{P}_{1-y}$ system^{4,5}. It has been shown that non-radiative recombination velocity near the mirror facet for the InGaAsP system is at least two orders of magnitude less than for AlGaAs system⁶. On the other hand, due to the presence of a big atom such as In, dislocation mobility in InGaAsP alloys is much less than in the AlGaAs system. Other advantages of InGaAsP systems include the possibility of selective etching and regrowth, which is essential for laser-diode fabrication. The absence of surface oxidation in this system facilitates the regrowth of chemically etched *mesa*-structures and gratings, eliminating the problems associated with the oxidation in AlGaAs-based system⁷.

The $\text{In}_{1-x}\text{Ga}_x\text{As}_y\text{P}_{1-y}$ compounds with $0.51 < x < 1$ and $0 < y < 1$, lattice-matched to GaAs, cover the same range of direct band-gap energy as AlGaAs (1.42 eV–1.92 eV)⁹.

Laser-quality material should combine close lattice-match to the substrate with excellent radiative and electrical properties. Fabrication of InGaAsP lasers on GaAs substrates has been hindered previously by the difficulties in epitaxial growth of high-quality multilayer structures. In principle, such problems can be overcome using low-pressure metal organic chemical vapour deposition (LP-MOCVD), which is now widely used in indus-

trial fabrication of semiconductor devices⁹. In this process, controlled amounts of group III alkyls and group V hydrides are introduced into a reaction chamber that contains a substrate positioned on a heated SiC-coated graphite block (susceptor). The hot susceptor has a catalytic effect on the decomposition of the gaseous products, and the growth therefore takes place primarily at this hot surface. This technique allows the growth of high-quality multilayers of semiconductor with extremely fine dimensional and compositional control¹⁰; here we apply the same approach to the growth of $\text{In}_{1-x}\text{Ga}_x\text{As}_y\text{P}_{1-y}$ -GaAs multilayer structures on a GaAs substrate.

In the first step, InGaP-InGaAsP-InGaP-GaAs double heterostructure (DH) lasers (Fig. 1a) emitting at 0.808 μm have been fabricated⁸ by this technology, for characterization of the

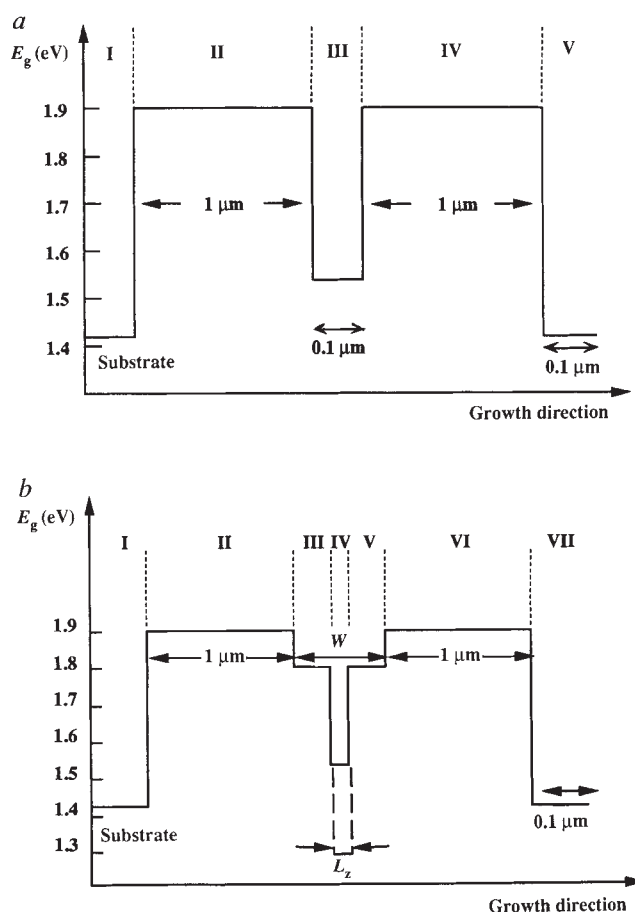


FIG. 1 a, Schematic diagram of the band structure of a double heterostructure (DH) InGaAsP laser emitting at 0.808 μm . Roman numerals indicate layers as follows: I, GaAs (001) substrate doped with Si (10^{18} cm^{-3}); II, $\text{In}_{0.49}\text{Ga}_{0.51}\text{P}$ confinement layer doped with silicon ($N_D - N_A = 10^{18} \text{ cm}^{-3}$); III, $\text{In}_{0.13}\text{Ga}_{0.87}\text{As}_{0.75}\text{P}_{0.25}$ active layer; IV, $\text{In}_{0.49}\text{Ga}_{0.51}\text{P}$ upper confinement layer doped with zinc ($N_A - N_D = 5 \times 10^{17} \text{ cm}^{-3}$); V, GaAs cap layer doped with zinc ($N_A - N_D = 10^{20} \text{ cm}^{-3}$). (E_g , band gap; N_A and N_D , acceptor and donor concentrations, respectively). b, Schematic diagram of the band structure of a separate-confinement heterostructure quantum well (SCH-QW) InGaAsP laser diode. I, GaAs (001) substrate doped with Si (10^{18} cm^{-3}); II, $\text{In}_{0.49}\text{Ga}_{0.51}\text{P}$ confinement layer doped with silicon ($N_D - N_A = 10^{18} \text{ cm}^{-3}$); III, V, $\text{In}_x\text{Ga}_{1-x}\text{As}_y\text{P}_{1-y}$ waveguide with thickness (W) varying between 0.2 and 0.5 μm ; IV, $\text{In}_x\text{Ga}_{1-x}\text{As}_y\text{P}_{1-y}$ active layer with thickness (L_z) varying between 100 and 500 Å; VI, $\text{In}_{0.49}\text{Ga}_{0.51}\text{P}$ upper confinement layer doped with zinc ($N_A - N_D = 5 \times 10^{17} \text{ cm}^{-3}$); VII, GaAs cap layer doped with zinc ($N_A - N_D = 10^{20} \text{ cm}^{-3}$). For the laser emitting at 0.8 μm , $x = 0.38$, $y = 0.25$, $x' = 0.13$ and $y' = 0.74$. For the 0.98- μm laser, the composition of the active layer corresponds to $x' = 0.2$, $y' = 1$ with $L_z = 40$ Å.

material. Broad-area contact laser diodes with a stripe width of 100 μm were prepared by a photolithographic technique. Measurements of absolute values of external efficiency for spontaneous emission, as well as the temperature dependence of emission efficiency, have shown that the internal efficiency of radiative recombination in the InGaAsP active layer for these laser diodes is close to 100% (ref. 8). This means that essentially all carriers injected in the $\text{In}_{1-x}\text{Ga}_x\text{As}_y\text{P}_{1-y}$ active region recombine radiatively at room temperature. The threshold current density for these diodes ($J_{\text{th}} \approx 700 \text{ A cm}^{-2}$) is close to the theoretical limit and better than that of the best DH AlGaAs/GaAs lasers with similar band diagrams ($J_{\text{th}} \geq 1,000 \text{ A cm}^{-2}$)¹¹.

In the second step, more advanced separate-confinement heterostructure quantum well (SCH-QW) laser diodes have been fabricated, which are suitable for pumping applications. The band diagram and structural details are shown in Fig. 1b. By changing the thickness and composition of the InGaAsP active layer, one can vary the emitting wavelength of these lasers between 0.7 and 1 μm . Due to the very thin active region (L_z in Fig. 1b) and quantum size effects, the energy distribution of the carriers is narrower in SCH-QW than DH lasers, with a thick active layer which leads to a further improvement in the main laser parameters.

As in the case of DH, broad-area lasers have been fabricated, and diodes were indium-bonded on copper heatsinks. The value of series resistance measured under a continuous wave (CW) regime for bonded laser diodes with 1 mm cavity length was as low as 0.04 Ω , which is three times less than for similar AlGaAs/GaAs lasers¹². Typical threshold current densities (J_{th}) as low as 220 A cm^{-2} , with differential efficiencies (η_d) as high as 1.1 W A^{-1} were obtained, for diodes with a cavity length of 1 mm and an emitting wavelength of 0.808 μm . For comparison, the best commercially available AlGaAs-based diodes emitting at 0.808 μm wavelength have $J_{\text{th}} = 300\text{--}400 \text{ A cm}^{-2}$, and $\eta_d = 0.5\text{--}0.8 \text{ W A}^{-1}$ (ref. 12). T_0 , the parameter characterizing the temperature dependence of the threshold current density ($J_{\text{th}}(T) = J_{\text{th}}(T_0) \exp(T/T_0)$) reached 175 K which is comparable with the best results obtained for AlGaAs (130–160 K, ref. 11).

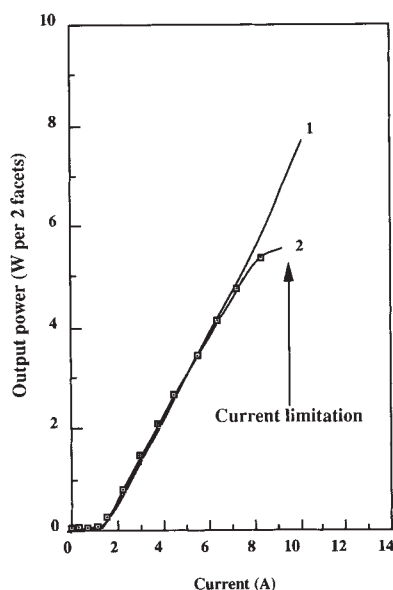


FIG. 2 Typical output power as a function of injection current for InGaAsP-GaAs SCH-QW lasers emitting at 0.8 μm under continuous wave (trace 2) and quasi-continuous wave (trace 1) operation. (Operating conditions: uncoated mirrors; quasi-continuous wave, pulse-width 200 μs , repetition rate 10 Hz; temperature T , 300 K; cavity length L , 1 mm.)

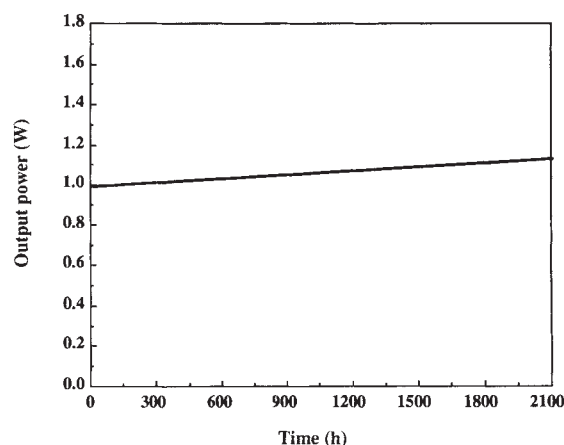


FIG. 3 1 W constant-power CW aging test at 50 $^{\circ}\text{C}$ for $\text{In}_{0.20}\text{Ga}_{0.80}\text{As}/\text{GaAs}/\text{In}_{0.49}\text{Ga}_{0.51}\text{P}$ SCH-QW lasers emitting at 0.98 μm . (T , 50 $^{\circ}\text{C}$; L , 1 mm; stripe width W , 100 μm .)

The distribution of radiation intensity across 100 μm of diode aperture was highly uniform for a wide range of driving current densities. The laser diodes were tested in both continuous wave (CW) and quasi-CW regimes (pulse width 200 μs , repetition rate 10 Hz), typical of Nd:YAG laser pumping applications. As shown in Fig. 2, the power obtained for quasi-CW was as high as 8 W, and for CW, 6 W (limited by the maximum driving current available in our setup), measured for uncoated mirror facets. This is more than an order of magnitude higher than that obtained from similar AlGaAs lasers with uncoated facets³.

Optical pumping applications impose additional requirements on high-power diodes, the first being the possibility of obtaining a specific emission wavelength. Our investigation showed that the lasing wavelength may be precisely adjusted to a desirable position by varying the cavity length and/or heatsink temperature of the laser diodes. The spectral full width at half maximum (FWHM) remained as narrow as 2 nm for currents up to 3–4 times the threshold current under pulse testing. This spectrum halfwidth is characteristic of highly uniform laser structures grown by MOCVD and molecular-beam epitaxy (MBE) and meets the requirements for solid-state laser pumping systems^{13,14}.

The FWHM of optical beam divergence in the direction perpendicular to the active layer is another important factor for pumping diodes. The value of this halfwidth for these lasers is 27 $^{\circ}$, which is significantly less than for pumping AlGaAs lasers (32–48 $^{\circ}$ divergence, ref. 12). The small difference in the refractive indices of waveguide and cladding layers in InGaAsP system is the reason for such a narrow beam divergence.

Preliminary lifetesting of InGaAsP (SCH-QW) lasers with uncoated facets emitting at 0.808 μm at 40 $^{\circ}\text{C}$ for more than 1,000 hours did not show any degradation. This device is currently running continuously without any further degradation. Equivalent results cannot be obtained with uncoated AlGaAs/GaAs lasers³.

Reliable high-power InGaAs/GaAs/InGaP single quantum well lasers emitting at 0.98 μm were also grown by LP-MOCVD. Threshold current density as low as 80 A cm^{-2} and differential efficiency as high as 75% were observed for 1-mm-long coated lasers. Device lifetime at 50 $^{\circ}\text{C}$ exceeded 2,000 hours at 1 W output power, with degradation less than 20% as shown in Fig. 3. The device is currently running continuously at 1 W at 50 $^{\circ}\text{C}$. Based on these results, the expected lifetime for Al-free lasers is $10^5\text{--}10^7$ hours compared to $10^4\text{--}10^5$ hours for AlGaAs-based³.

The main parameters of InGaAsP-based high-power laser diodes such as J_{th} , T_0 , η_d are comparable with the best results obtained with AlGaAs-based laser diodes. On the other hand,

the series resistance, beam divergence and resistance to degradation under high-power operation of InGaAsP-based lasers are superior to AlGaAs systems. The advantages of InGaAsP system mentioned above seem to be sufficient for replacing the AlGaAs-based lasers in present-day high-power applications, and may stimulate the search for new applications of near-infrared diode lasers. □

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Density-driven liquid–liquid phase separation in the system $\text{Al}_2\text{O}_3\text{--Y}_2\text{O}_3$

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PHASE separation of liquid mixtures into two liquids with different compositions is a well-known phenomenon. It has been proposed^{1–9} that another type of liquid–liquid phase separation, driven by fluctuations in density rather than in composition, may occur in some elemental systems. Transitions between low- and high-density amorphous phases have been described for the one-component oxides H_2O , SiO_2 and GeO_2 (refs 10–17), and it has been suggested^{18–21} that a liquid–liquid phase transition might occur in supercooled water. If density-driven phase separation truly does occur in liquid mixtures, it should be possible to observe the coexistence of two liquids with the same composition but different density. Here we report the direct observation of such a situation. We observe two coexisting liquid phases in the supercooled melt of $\text{Al}_2\text{O}_3\text{--Y}_2\text{O}_3$ just above the glass transition at ambient pressure, both of which have the same composition. We propose that these two phases must differ solely in density, and that the transition is entropically driven. The occurrence of the phase transition in this system may explain why the crystallization of yttrium aluminium garnet, the host material for Nd^{3+} ions in YAG lasers, is sluggish^{22–25}.

The occurrence of maxima in the melting curves of many metals, alloys and simple salts has been related to the presence of distinct structural species in the liquid, with relative proportions varying with pressure and temperature^{6,8}. Large changes in the electrical conductivity of molten metals and chalcogens with

pressure have also been analysed by a two-species model^{7,8}. Changes in the pair distribution functions of liquid Cs, Bi, Ga and Se suggest that the electrical conductivity variations are due to rapid changes in liquid structure, between low- and high-density states characterized by different coordination number and connectivity⁹. The volume change accompanying the semiconductor–metal transition in liquid Se suggests a first-order transition, implying two distinct liquid phases^{2,4}. Studies on liquid S suggest transitions between three liquid phases⁴. Analysis of available data for liquid C, along with results of calculations from first principles has led to a pressure–temperature (P – T) diagram with a first-order transition between liquids with graphitic and diamond-like structures⁵. Liquid H_2O and SiO_2 both exhibit temperature maxima in their densities^{26,27}. It has recently been argued for H_2O that the thermodynamic anomalies are related to critical behaviour associated with a transition between low- and high-density phases^{18–21}, in agreement with the observation of distinct low- and high-density forms of amorphous solid H_2O ^{10,28,29}. Similar arguments have been extended to SiO_2 and GeO_2 liquids and glasses, which exhibit reversible pressure-induced transitions between low- and high-density forms, associated with changes in coordination number^{11–17}.

The garnet $\text{Y}_3\text{Al}_5\text{O}_{12}$ (YAG) in the $\text{Y}_2\text{O}_3\text{--Al}_2\text{O}_3$ system is an important technological material^{22–25}. Single-crystal growth of YAG is rendered difficult by its sluggish crystallization from the melt, and by the extreme temperature sensitivity of the process. In the absence of seed crystals, or if the melt temperature exceeds a ‘critical’ value, metastable crystallization of YAlO_3 perovskite and alumina occurs over a range of compositions^{22–25}. Although this behaviour is primarily due to the relative growth and melting kinetics of the crystalline phases, it has sparked interest in the structural and thermodynamic properties of $\text{Y}_2\text{O}_3\text{--Al}_2\text{O}_3$ and related liquids^{22–25,30,31}.

We undertook a study of the $\text{Y}_2\text{O}_3\text{--Al}_2\text{O}_3$ liquid system by hot-stage microscopy. Metastable crystallization was verified near the composition of YAG, and the solidus and liquidus temperatures observed were in agreement with previous studies^{22–24}. The liquids could be supercooled to several hundred degrees below the liquidus (1,300–1,400 °C). No change in the appearance of the liquid was observed while the liquid was held in this temperature regime. Glasses could be quenched from the supercooled liquid with compositions between 24–32 mol% Y_2O_3 , by switching off the power to obtain a cooling rate of 300–400 degrees s^{-1} .

For all of the samples which formed glass, we observed a second liquid phase appear spontaneously throughout the bulk of the supercooled liquid sample during quenching. This second phase formed bubbles which grew during the quench, before both phases were frozen at the glass transformation temperature, T_g , giving rise to glassy inclusions in a glassy matrix (Fig. 1). Nucleation and growth of the second liquid phase were also observed at 20 and 22 mol% Y_2O_3 , but these samples crystallized during quenching, and could not be obtained in the glassy state. The volume fraction of the glassy inclusion phase in the sample increased with decreasing Y_2O_3 content, and with decreasing quench rate (Fig. 1b, c), but never reached 100% for the samples which could be quenched to glass. Attempts to grow larger inclusions via slower quenching or re-heating glassy samples failed due to crystallization, which precluded calorimetric study of the glassy phases. The liquid–liquid separation temperature increased slightly but systematically with increasing Al_2O_3 content (Fig. 2). The compositions of the two glassy phases present in each sample were determined by electron probe microanalysis (Table 1). To our surprise, the glassy matrix and inclusion phases were found to have identical compositions, within experimental error ($2\sigma < \pm 2$ relative atom%), for all bulk compositions studied.

Using primary backscattered electrons to image the sample, the inclusion phase always appeared darker than the matrix, indicating a lower average electron density within the inclusion

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(Fig. 1d). For phases with equal composition, this indicates a lower mass density for the glassy inclusions compared with their matrix. We have confirmed by optical microscopy, through observation of the direction of movement of the Becke line (the bright line of light which appears at the interface between materials of different refractive index), that the inclusions have lower refractive index than the matrix.

The lower density of the glassy inclusion phase, formed at lower temperature, gives an indication of the mechanism of the liquid-liquid transition. Because $\text{Y}_2\text{O}_3\text{-Al}_2\text{O}_3$ is a two-component system, 'conventional' phase separation into two liquids with different compositions might at first be invoked, with a narrow compositional gap corresponding to the small uncertainty in the electron probe analysis. However this is

unlikely, because the 'unmixing' was observed to occur over a wide range of bulk compositions, and in each case gave rise to matrix and inclusion phases with identical compositions.

As an alternative approach to understand our observation, we propose the existence of a metastable liquid-liquid P - T phase boundary, between two $\text{Al}_2\text{O}_3\text{-Y}_2\text{O}_3$ liquids with the same composition but different density, analogous to that suggested for water¹⁸⁻²⁰ (Fig. 2). This boundary is extended into a surface in the P - T - x (where x is composition) field of the system $\text{Al}_2\text{O}_3\text{-Y}_2\text{O}_3$, resulting in a curve, T_{1-1} , in the constant pressure T - x diagram (Fig. 2). The two liquids will coexist stably only at the phase transition temperature at a given pressure. In our quenching experiments, the higher-temperature liquid phase with higher density is retained metastably to temperatures below the coexist-

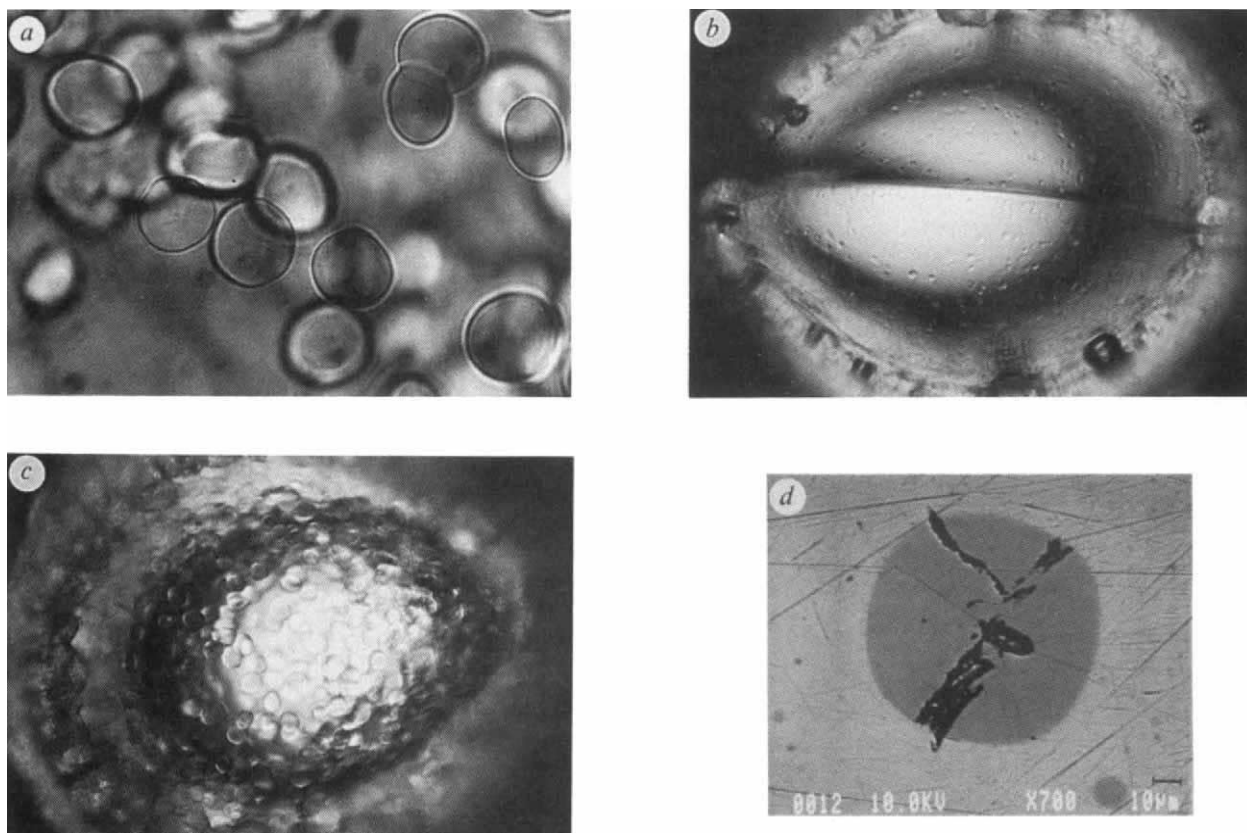


FIG. 1 a, Transmitted-light optical micrograph of a typical sample in the glass-forming region (30.1 mol% Y_2O_3), showing the glassy inclusion phase in a glassy matrix of the same composition. The field of view is $\sim 100 \times 150 \mu\text{m}$. All the samples containing two glassy phases were found to be isotropic under polarized light, and no crystalline peaks were observed in the X-ray diffraction patterns (careful scans were made in the region of the strong diffraction peaks of $\text{Y}_3\text{Al}_5\text{O}_{12}$, which is the only isotropic crystalline phase in the system). Starting materials in the composition range 20–37.5 mol% Y_2O_3 were prepared by a sol-gel synthesis³⁴, and heated by d.c. resistance heating of a metal wire in a water-cooled microscope heating stage³⁵⁻³⁷. To obtain the high temperatures required for this study, the microscope hot stage³⁵⁻³⁷ was modified by exchanging the Pt-Ir wire furnace with a pure Ir wire, operated in inert (N_2) atmosphere to avoid oxidation of the iridium. Powder samples were loaded into a 0.6-mm-diameter hole in the 1-mm-diameter Ir wire. The hot stage was placed under an optical microscope for visual examination of the samples during heating and cooling. Quenching of the liquids was achieved by rapidly turning the voltage down. Temperature was calibrated from series of known melting-point standards, including crystalline phases in the $\text{Y}_2\text{O}_3\text{-Al}_2\text{O}_3$ system. The temperatures at which the second liquid phase appeared, and the glass transition temperature (T_g) were estimated from the temperature at the beginning of the quench, the timed quench rate, and the calibrated

power to the Ir wire furnace. b, c, Transmitted-light optical micrographs of the same glassy sample, at lower magnification than a, showing effects of variable quench rate. Both micrographs show the glassy sample in the Ir wire, which appears as the dark material (towards the corners of the micrographs) surrounding the glass. The microscope is focused just below the surface of the glass, at some depth within the hole in the Ir wire. In both photographs, the hole in the Ir furnace wire is 0.6 mm in diameter. The horizontal line in b (faster quench) is a cooling crack which traverses the sample. The sample in b was quenched rapidly from 1,900 °C, by increasing the N_2 flow rate, and switching off the power to Ir wire furnace (400 degrees s^{-1}). The inclusions in this photograph range from 5–10 μm in diameter. Micrograph c represents the same sample, quenched more slowly by manually turning the power down, and clearly shows growth of larger (20–50 μm diameter), more numerous glassy inclusions than in b. d, Back-scattered electron (BSE) image of a glass-in-glass inclusion in the $\text{Y}_2\text{O}_3\text{-Al}_2\text{O}_3$ system. The matrix is lighter (brighter BSE image) because of higher mass density at constant composition. The black areas are due to cracking in the sample. (Scale bar, 10 μm .) For all samples studied, the composition of the matrix and the inclusion were the same within the uncertainty of the probe analysis (Table 1). For the sample shown here, both the inclusion and matrix phases had Y_2O_3 contents of $30.1 \pm 0.7 \text{ mol}\%$.

TABLE 1 Analysis of quenched $\text{Y}_2\text{O}_3\text{--Al}_2\text{O}_3$ sample

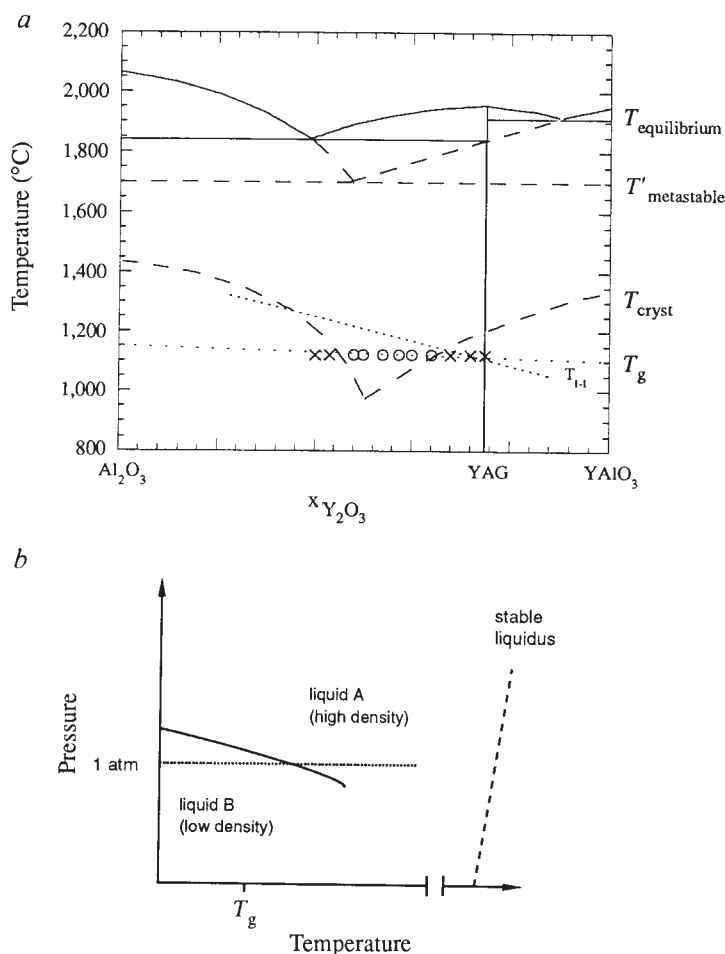
	Glassy inclusion phase			Glassy matrix		
	Y (at%)	Al (at%)	O (at%)	Y (at%)	Al (at%)	O (at%)
	11.65	28.59	59.76	11.43	28.54	60.03
	11.69	28.59	59.72	11.54	28.30	60.16
	11.44	28.73	59.83	11.50	28.62	59.88
	11.49	28.66	59.85	11.50	28.32	60.10
	11.64	28.24	60.12	11.45	28.43	60.12
	11.42	28.51	60.07	11.39	28.37	60.24
	11.31	28.96	59.74	11.29	27.83	60.88
	11.44	28.67	59.89	11.61	28.62	59.77
11.37	28.60	60.04	11.56	28.71	59.73	
11.61	28.11	60.29	11.46	28.26	60.28	
11.49	28.41	60.09				
11.46	28.49	60.05				
Mean	11.50	28.55	59.95	11.47	28.40	60.12
2σ	0.22	0.42	0.34	0.18	0.48	0.31

Electron probe microanalysis of a typical $\text{Y}_2\text{O}_3\text{--Al}_2\text{O}_3$ glassy inclusion and surrounding glassy matrix (Fig. 1), obtained in this study. Each sample was analysed for all three elements (Y, Al and O) by wavelength-dispersive analysis, using crystalline YAG as standard. Elemental totals were $100 \pm 1\%$ by weight. No additional elements were observed by energy-dispersive analysis. The diameter of the probe beam was of the order of $1\text{--}2\text{ }\mu\text{m}$, much smaller than the diameter of the inclusions analysed ($10\text{--}50\text{ }\mu\text{m}$). The individual analyses in this table represent both several points within a given inclusion, and points from several different inclusions, within the same sample. The compositions of the matrix and inclusion are identical, within analytical error (reported as 2σ of the mean analysis). 10–30 points were analysed for each sample.

FIG. 2 a, The $\text{Al}_2\text{O}_3\text{--Y}_2\text{O}_3$ equilibrium phase diagram is shown as a solid line, together with the metastable crystallization diagram (dashed line)^{22–24}. The glass-forming compositions found in this study are marked with open circles. The glass transformation temperature of the supercooled liquid (T_g) is estimated at $\sim 1,150^\circ\text{C}$, from optical observation of the sample freezing to glass, the temperature calibration of the furnace wire and the timed quenching rate (see Fig. 1 legend). T_g was not found to vary significantly with composition in this composition range, consistent with the relatively flat liquidus. The ratio of T_g to the melting temperature is $\sim 2/3$, consistent with many glass-forming systems³⁸. Compositions which did not quench to glass are indicated by crosses. The temperatures for homogeneous or heterogeneous crystallization (T_{cryst}) in our experiments are presumed to lie sub-parallel to the metastable melting line. The intersection of T_{cryst} with T_g defines the glass-forming region in our study. A higher quenching rate, or use of containerless conditions^{38–41}, would extend the glass-forming region and thereby probably the region where we can find a second liquid phase. The line T_{l-1} indicates the temperature at which the second liquid phase is observed to nucleate (measured during the quench by the timed quenching rate, and the calibrated furnace power), and decreases with increasing Y_2O_3 content. Our experimental observation that the volume fraction of the low-temperature phase increases with decreasing Y_2O_3 content, also indicates that the separation between T_g and T_{l-1} increases with decreasing Y_2O_3 content. b, The proposed metastable liquid–liquid transformation line in the $P\text{--}T$ plane (at fixed composition within the $\text{Y}_2\text{O}_3\text{--Al}_2\text{O}_3$ system), drawn by analogy with the suggested phase behaviour of supercooled liquid water^{18–21}, which gives a thermodynamic rationalization for our experimental result. The two liquids have the same composition, but different densities. The high-temperature liquid, corresponding to the glassy matrix phase in Fig. 1, has higher density than the low-temperature liquid which gives rise to the glassy inclusions. The high-temperature, high-density (matrix) liquid also has higher entropy (Fig. 3), so that the Clapeyron slope ($dP/dT = \Delta V/\Delta S$) is negative. The observed liquid–liquid phase separation is quite likely to be general for such A_2X_3 systems. In our case, it was fortunate that the liquid–liquid coexistence line intersected our cooling line (dotted) above T_g (and above the critical temperature for crystallization) at room pressure, so that it could be observed and the samples quenched to glass in our experiments.

ence line. The lower-temperature (lower-density) liquid phase is observed to nucleate and grow within the supercooled high-density liquid matrix to form the observed inclusions, consistent with a first-order liquid–liquid transformation process. The growth rate in such processes is strongly dependent on the liquid viscosity. The measured viscosity of molten YAG shows Arrhenius behaviour³⁰. Extrapolation to a glass transition in the neighbourhood of $1,150^\circ\text{C}$ gives a viscosity of 10 poise (P), rather than the necessary large value near 10^{13} P at T_g , so that the viscosity must increase rapidly in the temperature range just above T_g . It is reasonable that the liquid–liquid transformation is arrested if the transition temperature is close to T_g , and if the system is cooled rapidly through this temperature region. Attempts were made to slow the cooling rate sufficiently to allow complete transformation from the high-temperature to the low-temperature liquid phase, but this was always intersected by nucleation of crystalline phases.

We obtained micro-infrared and Raman spectra separately from the matrix (high-temperature, high-density) and inclusion (low-temperature, low-density) glassy phases (Fig. 3). The spectra of each are quite different, and show little variation with bulk composition. The spectrum of the matrix glass is quite featureless, compared with that of the glassy inclusions, which shows several broad bands (Fig. 3). This indicates that the matrix glass is more structurally disordered, that is, has a higher entropy, than the inclusion phase. Combined with the higher density of the high-temperature matrix phase, this is consistent with the negative Clapeyron slope ($dP/dT = \Delta V/\Delta S$, where ΔV and ΔS are the volume (V) and entropy (S) differences between the two liquids) proposed for the transformation (Fig. 2).



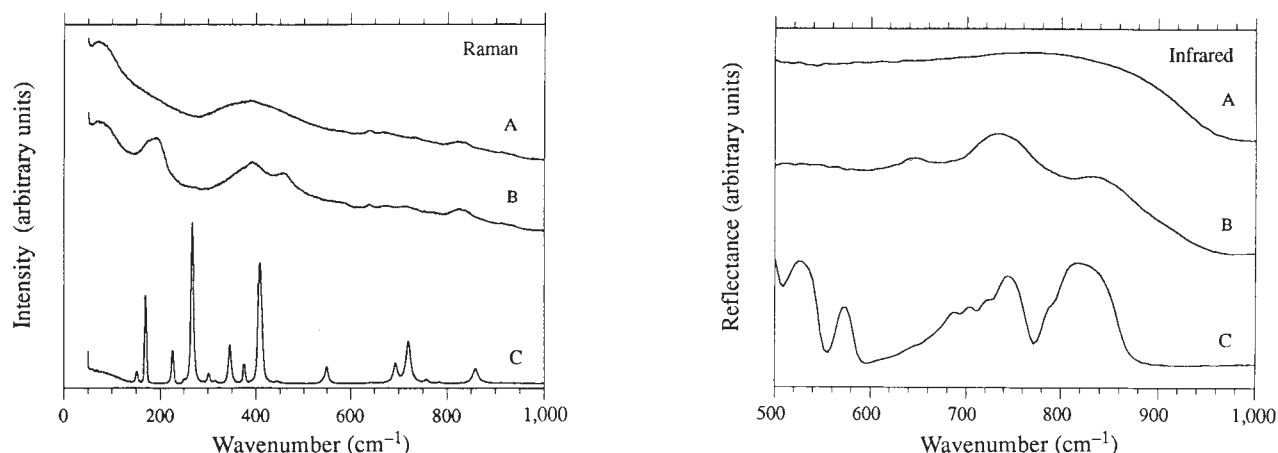


FIG. 3 Raman (left) and infrared (right) spectra of the glassy matrix (A) and glassy inclusion (B) phases from a typical sample, together with the spectra of single crystal YAG^{42,43} (C). The spectra of the glassy compounds are clearly different from the spectrum of single-crystal YAG, confirming that the inclusions are not crystalline YAG, which is the only optically isotropic crystalline phase in the system. The infrared and

Raman spectra of the inclusion contain more structure than the matrix, indicating that a structural change to a more ordered liquid has taken place. There are only minor changes in the vibrational spectra of the glassy inclusion and matrix phases with composition, over the range studied.

We can generally relate the liquid density behaviour to structural changes in the solid state. Crystalline YAG has been shown to decompose to YAlO_3 and Al_2O_3 at pressures around 40 kbar at 1,000 °C (ref. 32). The four-coordinated Al^{3+} ions in the garnet change to six-coordination, in YAlO_3 and Al_2O_3 , and the overall density is increased. If the supercooled liquid is examined at constant temperature (Fig. 2), the lower-density liquid probably has a smaller average Al coordination than the high-density liquid phase, by analogy with the crystalline phase transition.

A structural interpretation for the thermal liquid-liquid transformation can be gained by considering the oxygen environments in corresponding crystals. In the garnet crystal, the O^{2-} ions all have the same coordination environment (OY_2Al_2). In crystal-

line YAlO_3 and Al_2O_3 , several different O^{2-} environments are encountered (OAl_4 , OY_3Al_2 , OY_2Al_2)³³. If the low-temperature liquid, like crystalline YAG, has a small number of distinct oxygen environments, it will have lower entropy than a high-temperature liquid with a greater range of oxygen sites.

This observation could help explain the sluggish crystallization of YAG, with a structural requirement of a single type of oxygen site, from the high-temperature melt, which corresponds to the high-entropy liquid phase. In addition, because crystalline YAG melts to the high-entropy liquid, the resulting large $-\Delta S$ term associated with melting could be responsible for depressing the YAG liquidus, which is nearly flat in the region of the garnet composition^{22, 24}. □

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Efficient manganese catalysts for low-temperature bleaching

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THE detergents of the next century will be routinely required to contain bleaching agents that are not only more active than those currently available but also environmentally safe and cost-effective. Hydrogen peroxide, the traditional bleaching agent¹, loses its activity as the washing temperature decreases. Peroxyacetic acid maintains acceptable bleaching activity down to 40–60 °C (ref. 2), but still lower temperatures are desirable. It is generally recognized that manganese and iron complexes are less environmentally damaging reagents than other transition-metal compounds, and such complexes have received considerable attention as bleaching catalysts^{3–11}. Here we show that manganese complexes derived from 1,4,7-trimethyl-1,4,7-triazacyclononane and related ligand systems act as highly effective catalysts for the bleaching of stains by hydrogen peroxide at low temperatures. These complexes also catalyse the epoxidation of alkenes and the oxidation of polyphenolic substrates by hydrogen peroxide. Our results demonstrate the considerable potential of these systems for clean and efficient low-temperature bleaching.

The manganese complexes 1–8 used here are derived from either 1,4,7-trimethyl-1,4,7-triazacyclononane (L^1) or the substituted triazacyclononane ligands (L^2 and L^3) (Fig. 1). To compare the different Mn complexes for stain oxidation, model bleaching experiments with tea-stained test cloths (commercially available as BC-1 cloths) have been performed. These test cloths are particularly suitable as monitors for bleaching because they show little response to detergency.

Manganese salts show a modest catalytic bleaching benefit in comparison to the use of H_2O_2 alone (Table 1). However, 'free'

manganese salts are not a viable option in detergents because their high instability leads to the formation of brown stains on textiles due to precipitation. In contrast, a very high catalytic bleaching effect was observed with $[Mn(IV)_2(\mu-O)_3L^1](PF_6)_2$ (1), $[Mn(III)Mn(IV)(\mu-O)_2(\mu-CH_3COO)L^1](PF_6)_2$ (2) and $[Mn(III)L^1(acac)(H_2O)](ClO_4)_2$ (3) compared to free Mn salts (Table 1), where $\mu-O$ and $\mu-CH_3COO$ means bridging oxygen and acetate respectively; acac, acetylacetonate (2,4-pentanedionate). The amount of unreacted H_2O_2 in solution at the end of the bleach with 1–3 was found to be $\geq 60\%$. Similarly, other compounds 4–7 containing the MnL^1 moiety^{12–14} also showed excellent catalytic bleach enhancement. Interestingly, a similar superior bleach activity was observed with a combination of free manganese salts and L^1 , added separately to the bleach solution. Many other manganese complexes^{5–10} have been tested for their bleaching activity as well. However, it was observed that Mn complexes without the triazacyclononane nucleus show a much smaller bleaching activity than compounds 1–7 at pH 10. This is attributable to their instability under the bleaching conditions.

The pH dependence of the hydrogen peroxide bleaching catalysed by 1, 2, and 3 is shown in Fig. 2. Compounds 1 and 3 show a higher catalytic activity for bleaching than compound 2 at pH < 9.5. Conversely, the catalysed bleaching of 2 is much better than that of 1 or 3 at pH > 9.5.

Model oxidation studies were performed in solution using polyphenolic compounds and alkenes. Polyphenolic compounds, which are appropriate models for tea stains, can be oxidized with hydrogen peroxide. Compound 2 has been examined as a test catalyst along with 1 and 3 for the homogeneous oxidation, because compound 2 retains a dinuclear core at all pH values (see below). Not surprisingly, ultraviolet-visible spectroscopy of the L^1 -containing compounds 1–3 revealed complicated kinetic behaviour, as these compounds form a mixture of mono- and dinuclear species at low pH. When $2.5 \mu\text{mol l}^{-1}$ of 2 was added to such a solution, the rate of oxidation at pH 10 increased by several orders of magnitude. At higher pH values (pH > 10), a still faster reaction was observed with 2 compared to 1 and 3, a trend reminiscent of that observed in the BC-1 bleaching experiments already described (Fig. 2).

The epoxidation of 4-vinylbenzoic acid (a water-soluble alkene) and styrene (a non-water-soluble alkene) with H_2O_2 catalysed by a representative set of the manganese complexes revealed that all complexes containing L^1 , L^2 and L^3 exhibit excellent catalytic activity (Table 2). The same epoxidation activity of $[MnL^2]PF_6$ (8)¹⁵ was observed after adding a fresh aliquot

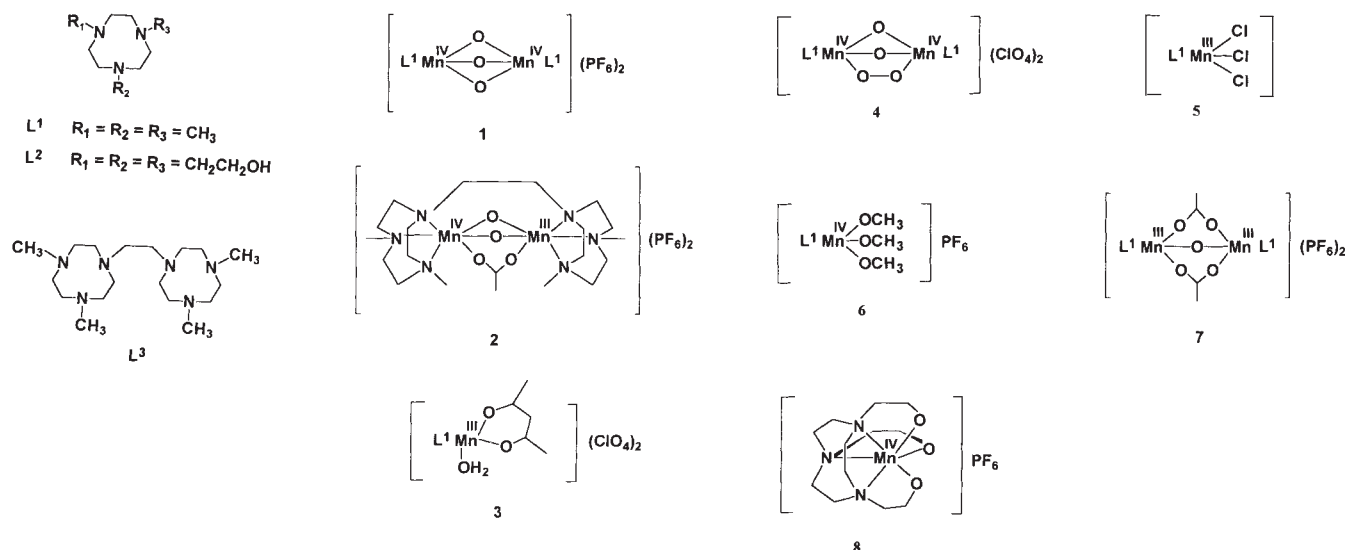


FIG. 1 Structures and abbreviations of the ligands and manganese complexes discussed in the text.

TABLE 1 Model bleaching experiments with BC-1

System	ΔR_{460} value
8.6 mmol l ⁻¹ H ₂ O ₂	6
H ₂ O ₂ with 5 μ mol l ⁻¹ MnSO ₄	12
H ₂ O ₂ with 2.5 μ mol l ⁻¹ compound 1	27
H ₂ O ₂ with 5 μ mol l ⁻¹ compound 3	26
H ₂ O ₂ with 2.5 μ mol l ⁻¹ compound 2	30
9.0 mmol l ⁻¹ peroxyborate with 1.7 mmol l ⁻¹ TAED*	12

All bleaching experiments were carried out at 40 °C for 30 min in demineralized water at pH 10 with two pieces of tea-stained cloth (BC-1) in the absence of a detergent formulation. Before and after the bleaching experiments the reflectance (R) of the tea-stained cloths was measured at 460 nm. The difference, ΔR_{460} , is a measure of the bleaching performance, that is, the removal of tea soil from the cloths (a higher ΔR_{460} value means a better bleaching performance).

* This is the prevailing system in European detergent powders, and generates 3.4 mmol l⁻¹ peroxyacetic acid.

of 4-vinylbenzoic acid at the end of the experiment described in Table 2. This procedure could be repeated at least four times without any loss of activity, showing that the catalytic system is quite stable. Further, the epoxidation of 4-vinylbenzoic acid with H₂¹⁸O₂ and **8** gave a product with 100% ¹⁸O, suggesting that the oxygen of the epoxide is derived from hydrogen peroxide and not water. In the past, high-valent metal-oxo species have been proposed as active intermediates in epoxidation reactions^{10,11} with manganese complexes as well as with mono-oxygenases. It appears that the intermediacy of manganese-oxo or manganese-peroxo intermediates does not seem unreasonable even with this type of epoxidation catalysts. Interestingly, although **8** exhibits a high epoxidation activity, no stain bleaching on BC-1 cloths was observed between pH 7 and 10. The optimum pH for epoxidation was established to be around 8.0, whereas the optimum pH for bleaching was found to be around pH 10. Thus it appears that catalytic epoxidation and fabric bleaching involve different mechanisms.

All compounds containing the MnL^I structure produce excellent bleach catalysis at pH 10, suggesting the formation of similar active species. The mechanism of catalysed bleaching was

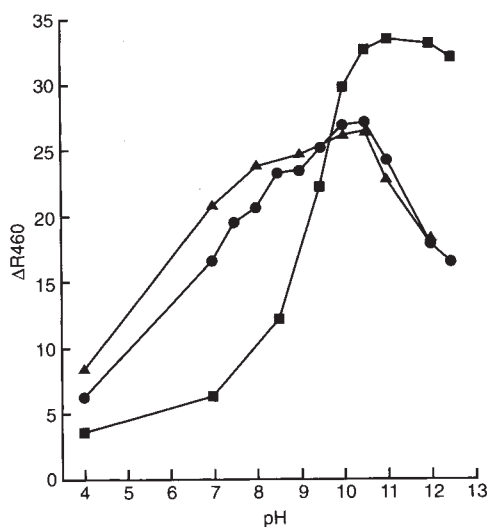


FIG. 2 The bleaching performance of compounds **1–3** as a function of pH (experimental procedure as for Table 2). (●) Compound **1**; (■) compound **2**; (▲) compound **3**.

investigated by electron paramagnetic resonance spectroscopy (EPR) in aqueous solutions. [Mn(IV)₂(μ-O)₃L₂](PF₆)₂ (**1**) is EPR silent at 77 K. The strong antiferromagnetic coupling (spin pairing) between the 3d electrons of each manganese ion leads to a diamagnetic ground state¹². No detectable change was observed in the ultraviolet-visible or EPR spectra of **1** when hydrogen peroxide was added at pH 10. Also, no ¹⁸O-exchange reactions could be detected on mixing [Mn(IV)₂(μ-¹⁸O)₃L₂](PF₆)₂ and peroxyborate solution, confirming the lack of reaction between the catalyst and oxidant. As tea stains contain polyphenolic chromophores that can be oxidized¹⁶, catechol was considered as an appropriate stain-mimic. The addition of catechol to an aqueous solution of **1** afforded an EPR spectrum with 16 peaks (Fig. 3), which is typical of a mixed-valence Mn(III)Mn(IV) complex with a coupling between the two ions^{5,17}. A similar 16-line spectrum was observed upon adding **1** to an aqueous solution containing tea. These results suggest that compound **1** also reacts with the tea chromophores via electron transfer yielding a mixed-valence Mn-complex. A 16-line EPR spectrum was also observed for each of the L^I-containing Mn complexes **3–7** in peroxyborate solutions at pH 10. In these cases, lower-valent manganese ions can form the Mn(III)Mn(IV) species by reaction with peroxyborate. Dissolving L^I and MnSO₄ in peroxyborate at pH 10 also yielded the same 16-line EPR spectrum. Compound **2** also showed a similar 16-line EPR spectrum as it is already a Mn(III)-Mn(IV) species. The relative intensities of the spectra observed on dissolving identical amounts (0.1 mmol l⁻¹) of **2** or **7** in borate solution at pH 10 are similar, suggesting that the amounts of mixed-valence Mn-species under these conditions are comparable. These solutions are stable towards decomposition (no change in intensity of the 16-line EPR spectrum even after 1 h at 40 °C). However, it was observed that the 16-line signal is much more stable in the case of **2** and **7** at pH 12. Thus, the higher bleach performance of compound **2** compared to L^I-containing compounds **1** and **3** at high pH (Fig. 2) appears to be related to the stability of this mixed-valence Mn species.

Although compound **1** has been found to be excellent for fabric stain bleaching at pH 10, this species could not be detected in the actual bleaching experiments by ultraviolet-visible spectroscopy or high-performance liquid chromatography. Modelling studies conducted in the case of **2** revealed that a

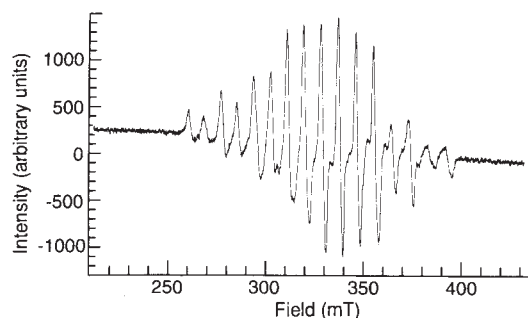


FIG. 3 Electron paramagnetic resonance spectrum of compound **1** with catechol in acetonitrile/borate solution at pH 10 after 10 min. METHODS. 1 mmol l⁻¹ **1** and 1 mmol l⁻¹ catechol were dissolved in acetonitrile/aqueous borate solution at pH 10. The solution was placed in an EPR tube and frozen in liquid nitrogen (77 K). The measurements were made using a Jeol JCS-RE2X spectrometer equipped with a Jeol Esprit 330 data system.

TABLE 2 Catalysed epoxidation of styrene and 4-vinyl benzoic acid

Compound	Styrene in methanol/buffer*		Vinylbenzoic acid in buffer†	
	Reaction time (h)	Conversion (%)	Reaction time (h)	Conversion (%)
No catalyst	24	< 2	4.3	19
MnCl ₂ ·6H ₂ O	24	55	5	92
1	5	99	2	98
2	3‡	99		
6	5	99	1.5	98
8	9	99	1.3	98§

* To a solution of 1 mol l⁻¹ H₂O₂ in methanol/carbonate buffer, pH 9.0 (1/3), 10 mmol l⁻¹ of styrene and 0.1 mmol l⁻¹ of the catalyst were added. The reaction mixture was stirred at room temperature and formation of the epoxidation product was monitored using HPLC and ¹H NMR spectroscopy.

† Oxidant, substrate and catalyst (10,000:100:1) were reacted in carbonate buffer at pH 8; reaction products were analysed using HPLC. A Hewlett-Packard 1050 instrument equipped with a 1040A diode array detector and a Regis ODS II 15-cm reverse column was used to analyse the reaction products. The eluent was a mixture of acetonitrile/phosphate buffer, pH 3 (3:1).

‡ After 1 h, because all H₂O₂ was decomposed, more was added.

§ The rate of epoxidation is pH-dependent, as more than 95% epoxide is formed after 5 h at pH 7.0, 60 min at pH 7.5, 75 min at pH 8.0, 5 h at pH 9.0, and 77 h at pH 10.0 (only 10% yield in this last case).

Mn₂(μ-O)₃ core structure is energetically highly demanding, and hence is unlikely to be involved in the actual bleaching process. Perhaps, in the case of **1** the Mn₂(μ-O)₃ core structure is not maintained during the bleach process for a different reason. A major difference between the L¹-containing complexes **1**, **3–7** and compound **2** is that although the catalysed bleaching capabilities of these complexes are very similar at pH 10, the L¹Mn complexes have clearly superior bleaching properties compared to **2** at pH < 9.5. Interestingly, the formation of mononuclear Mn(IV) species has been found by EPR⁵ only with **1** and **3–7** at pH < 9.5. Thus, it may be suggested that catalytic fabric bleaching involves mononuclear species at pH < 9.5, and dinuclear species above this pH. EPR experiments on **2** have revealed that the mixed-valence species is unaffected by the addition of catechol, whereas the addition of oxidant yields the EPR-silent Mn(IV)(μ-O)₂Mn(IV) species¹⁸. This species subsequently participates in the oxidation of stains. □

Pollen in marine sediments as an indicator of oxidation of organic matter

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ORGANIC carbon burial in marine sediments generates virtually all atmospheric oxygen, and provides a long-term sink for about 20% of all carbon^{1,2}; it is therefore important to understand the mechanisms controlling organic carbon preservation. There is a fraction of organic matter that is preserved under reducing conditions but which can be rapidly oxidized if exposed to molecular oxygen^{3–6}. It is not clear, however, how much of the total organic matter preserved in marine sediments is oxygen-sensitive. Here we present results from a relict turbidite in the Madeira abyssal plain which suggest that pollen grains can be used as a sensitive tracer of oxygen-sensitive organic carbon. We find that pollen grains were completely degraded within 10 kyr in the presence of diffusively introduced oxygen, but were well preserved for at least 100 kyr under anoxic conditions. We also present pollen data from the Pacific Northwest continental shelf, which suggest that oxic degradation can explain the decrease in organic carbon with depth commonly observed in coastal sediments.

The factors that lead to the preservation of organic matter (OM) in the marine sedimentary record remain the source of great debate. Many regulating factors are interrelated⁷, making discrimination between simultaneous processes difficult. Of the OM that reaches marine sediments, less than 50% can be identified at the molecular level⁸, and typically less than 10% is preserved⁹. Preservation is correlated with sedimentation rate¹⁰ and primary productivity¹¹, fostering hypotheses that OM preservation is controlled by either of these two factors. However, regions of high productivity and/or high sedimentation are often accompanied by bottom waters of low oxygen content¹², and most continental margin sediments become reducing within a few millimetres of their surface². The presence of large amounts of OM in these sediments could thus be a consequence of incomplete degradation under anoxic conditions, and this hypothesis is often cited as the controlling factor in the formation of carbon-rich sediments over long time periods¹². Oxygen effects have been difficult to directly define because (1) redox conditions of sediments are often altered during postdepositional events making indirect estimates from redox-sensitive elements hard to interpret⁴, (2) many processes concurrently affect sedimentary organic carbon concentrations^{2,7}, (3) rates of aerobic and anaerobic degradation in modern marine sediments are not significantly different¹³, and (4) a direct, readily measured tracer of oxygen-sensitive organic matter has been lacking¹.

In order to investigate definitively the 'preserved only under anoxia' hypothesis, it is necessary to find an ubiquitous tracer of known source whose degradation can be readily understood, and an experimental setting where oxygen availability is the only factor affecting preservation. Pollen grains within a Madeira abyssal plain turbidite (located in the northeast Atlantic Ocean under a water depth of ~5,400 m) provide such an opportunity. The f-turbidite, a 4-m-thick bed composed of sediment originally deposited along the northwest African coast (~700 km to the southwest), was mobilized and redeposited ~140 kyr before present (BP)^{3–6}. After turbidite flow, which represents essentially instantaneous deposition, molecular oxygen partially diffused down through the sediment for ~10 kyr and oxidized organic matter within the upper 50 cm (refs 3–6). The lower portion of the sequence remained reducing, and is thought to have con-

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tained abundant sulphate for over 100 kyr (refs 4, 5). Sediments within the top 3.75 m of the f-turbidite consist of equal and ungraded mixtures of silt-sized quartz, clays and coccolithophore shells⁴⁻⁶. The two sampling sites (core P5: 32° 2.50' N, 24° 12.50' W; core P25: 30° 44.23' N, 25° 22.45' W), located ~100 km apart, represent spatially distinct but mineralogically and geochemically similar subsamples of this extensive (~126 km³)⁶ turbidite.

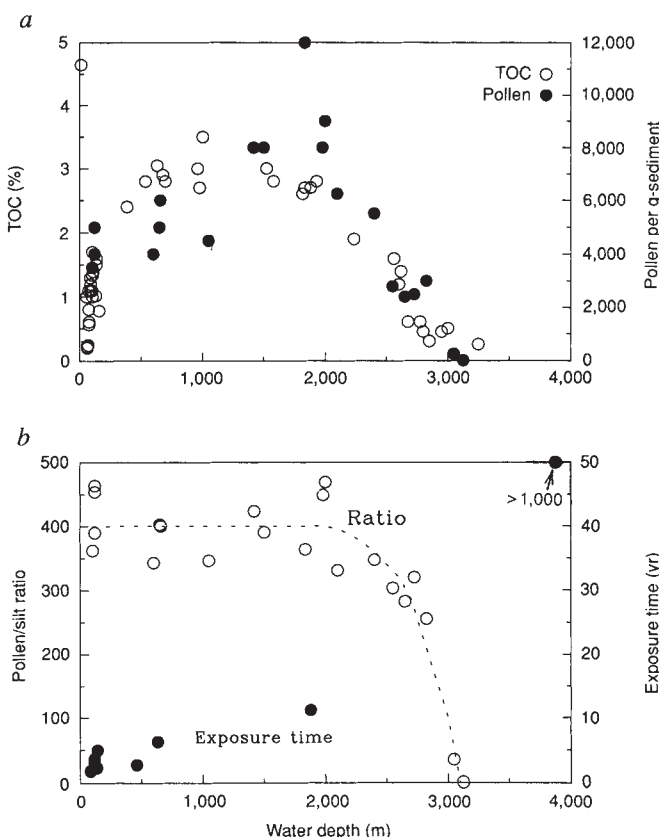
Total pollen concentrations and compositions in the lower unreacted portions of each core show excellent agreement (Table 1), in accord with the homogeneity of total OM³, alkenones⁶, and mineralogy^{4,5} previously observed within the f-turbidite. At stations P5 and P25, average pollen concentrations were $1,440 \pm 80$ and $1,740 \pm 10$ g⁻¹, respectively (Table 1). The slightly higher concentration found in P25 agrees with the small increase in total OM and slight decrease in sediment mean grain size⁴ between the cores. These differences have been attributed to variations in deposition during turbidite flow³⁻⁶. Pollen in unreacted portions of the core are generally well preserved, showing no visible signs of degradation of the exterior sporopollenin coat. *Chenopodiaceae*-*Amaranthaceae* (goosefoot-amaranth families), *Gramineae* (grass family), *Cyperaceae* (sedge family) and *Artemisia* (sagebrush) dominate the pollen assemblage, which derives mainly from steppe/desert plant communities. Similar pollen assemblages have been found in shallow marine sediments of the same age from along the northwest African coast¹⁴⁻¹⁶. These data indicate both the number and types of pollen initially present throughout the f-turbidite. As the interval sampled in each core spanned over 50% the unreacted portion of the turbidite, we conclude that although there are measurable differences between P5 and P25, within-site variability is small. Thus, samples below the oxidation front should closely approximate the pollen composition to be expected in the overlying oxidized layer at each site were it not oxidized.

In contrast to the unoxidized layers, there were no identifiable pollen grains or fragments in the oxidized layers from either

TABLE 1 Pollen data in cores from the Madeira abyssal plain

Core layer	Site P5			Site P25		
	Oxid.	Un.	Un.	Oxid.	Un.	Un.
Core depth (m)	7.10	7.55	7.77	7.85	8.60	9.15
Total organic carbon ³ (%)	0.16	0.93	0.94	0.21	1.00	1.02
Pollen type	Composition (% of total pollen)					
<i>Chenopodiaceae</i> - <i>Amaranthaceae</i>	0	26.9	26.5	0	25.5	18.9
Other periporate	0	5.8	12.2	0	9.9	10.7
<i>Gramineae</i>	0	14.7	13.8	0	18.0	24.5
<i>Cyperaceae</i>	0	19.2	17.1	0	11.2	16.4
<i>Artemisia</i>	0	9.0	5.5	0	7.5	5.0
Other Compositae	0	1.3	1.1	0	1.9	0.6
<i>Ephedra</i>	0	5.8	7.7	0	3.1	2.5
<i>Alnus</i>	0	0.6	0.0	0	0.6	0.0
<i>Pinaceae</i>	0	1.9	2.2	0	0.6	1.9
<i>Juniperus</i>	0	0.6	0.6	0	0.0	0.0
Monolete spores	0	1.3	0.6	0	0.6	0.0
Trilete spores	0	0.0	0.6	0	0.6	0.0
Unidentified	0	12.8	12.2	0	20.5	19.5
Total pollen/spores per gram sediment	0	1,380	1,510	0	1,760	1,720
Average		1,440			1,740	

Data from cores taken from the f-turbidite sequence of sediments in the Madeira abyssal plain. The f-turbidite is ~4 m thick, located under ~7 m of additional sediment⁴. Pollen were prepared according to standard techniques²⁹ and by sieving through a 7- μ m screen³⁰. *Lycopodium* spore tablets³¹ were added to calculate absolute concentrations. At least 150 grains were counted for each sample. (Oxid., oxidized layer; un., unreacted portions of core).



site, indicating that pollen grains from over 10 plant taxa were completely destroyed as a consequence of oxygen exposure (Table 1). All pollen, and 80% of the total organic matter^{3,6} that persisted for $>10^5$ yr in the presence of pore-water sulphate, was destroyed within 10^4 yr by exposure to molecular oxygen. The lost pollen encompass grain types both resistant and susceptible to experimental oxidation^{17,18}. These data clearly indicate that molecular oxygen is necessary to oxidize pollen grains in marine

FIG. 1 a, Pollen concentrations^{20,21} (F.S.H., unpublished data) and total organic carbon (TOC) (refs 23-25; A. Devol, unpublished data; R.G.K., unpublished data) in Pacific Northwest margin sediments as a function of water depth. Assuming that pollen grains contain an average of 10 ng-C each, pollen contributes ~1% of the organic matter present in this environment. b, Ratio of total pollen concentrations to percentage small silts (3-38 μ m), and times of exposure to oxygen, for sediments from the Washington shelf and slope (refs 20-22; R.G.K. and E. A. Presley, unpublished data). Estimates of oxygen exposure time were calculated as the oxygen penetration depth²³⁻²⁶ times the sedimentation rate²³ after accounting for changes in porosity²⁵, irrigation² and bioturbation²⁵. No data for oxygen exposure is available below 2,000 m from along the Washington coast. Exposure times in open ocean sediments are $>1,000$ yr (refs 2, 32), indicating that a sharp increase in oxygen exposure must occur between 2,000 and 5,000 m water depth.

sediments. As no pollen was found in the oxidized samples from the Madeira abyssal plain, a conservative estimate of the half-life of pollen in the presence of molecular oxygen can be made. Given 10 kyr of oxygen exposure, a final penetration depth of 50 cm, and a constant penetration rate⁴, the rate of oxygen penetration is $\sim 0.005 \text{ cm yr}^{-1}$. Our sample interval was within 10 cm of the bottom of the oxidized layer, indicating that the minimum time of exposure to oxygen was 2 kyr. Assuming first-order kinetics, pollen in these samples has a maximum half-life in oxic marine sediments of $\sim 300 \text{ yr}$ and a decay constant of at least 0.002 yr^{-1} . Consequently, pollen grains are potential indicators of prolonged oxygen exposure in marine sediments.

The potential of pollen as a palaeoxygenicity indicator has not been widely acknowledged, even though the 'oxygen-sensitivity' of pollen is qualitatively known to palynologists¹⁹. Pollen grains are found within the sedimentary record throughout the past 400 Myr (ref. 19), providing an extensive potential record of oxygen exposure, but to interpret this record, factors other than oxic degradation that affect pollen need to be addressed. Pollen concentrations and distributions in the marine environment should be affected primarily by three factors: productivity of the terrestrial source, delivery to and hydrodynamic distribution within the marine regime, and degradation. In estimating the extent of long-term exposure to oxygen, complicating factors could be minimized by only comparing pollen of similar age and source, and by comparing concentrations of pollen grains to the hydrodynamically-equivalent small clastic silt (8–38 μm) component, with which pollen are often dispersed²⁰. Lack of pollen in marine sediments with a fluviially-delivered silt component could therefore indicate long-term exposure to oxygen. The oxidized portion of the samples from the Madeira abyssal plain represents an example of this extreme situation, as do most modern, deep slope deposits (>3,000 m), which lack abundant pollen^{20,21} although they often have a significant 8–38 μm clastic silt component²².

To test this inference, data from modern surface sediments of the Pacific Northwest shelf and slope region^{7,20–26} were compiled, and estimates of pollen/silt ratios, along with estimates of oxygen exposure time, were plotted as a function of water column depth (Fig. 1). Similarities in pollen and OM concentrations suggest influence by common factors (Fig. 1a). The constant pollen/silt ratio on the shelf and upper slope supports the supposition that pollen grains and silts are introduced in this region via a riverine source^{7,20,21}, and transported together to depths of up to 2,500 m. The drop in pollen abundance in the deeper slope samples indicates that either the pollen never reached the deep slope, even though silts do, or that pollen was removed after

deposition. In support of the second hypothesis, there is no apparent reason that pollen and clastic silts should abruptly cease to exhibit hydrodynamic equivalency at depths >2,500 m. Also, exposure times of sedimentary pollen to oxygen increase dramatically below 2,500 m (Fig. 1b and caption), and the concentration of total organic matter typically decreases (Fig. 1a) as water depth increases. Many pollen grains are also typically visibly weathered at deeper oceanic sites^{21,27}. As the samples from the Madeira abyssal plain indicated that pollen grains are only destroyed under oxic conditions, the length of exposure to oxygen must have determined the distribution of pollen (and other oxygen-sensitive organic matter) in these Pacific Northwest margin sediments.

Chemical studies of oxic-degradation within single pollen types, in conjunction with other measurements, such as pollen/silt ratios, may provide complementary information on partial oxic degradation. For example, moderate exposure to oxygen of certain susceptible pollen types should lead to selective losses that are unlikely to result from climatic or transport-induced changes, as well as to selective changes in the chemical composition of sporopollenin from different species^{17,18}. In addition to alleviating the burden of showing that pollen grains were present before oxic-exposure destroyed them, single grain or species characterizations would be less sensitive to source or transport changes than total pollen counts and might reflect relatively brief periods of oxygen exposure.

Oxygen-sensitive degradation of organic matter may play a role in modulating atmospheric oxygen concentrations over geological time^{1,2}. In this model, higher atmospheric oxygen concentrations lead to greater oxygen concentrations in the ocean, larger diffusive fluxes of oxygen into sediments and more extensive *in situ* organic-matter oxidation. Negative-feedback control of oxygen concentrations should result via decreased organic carbon burial, increased oxygen consumption and ultimately decreased atmospheric oxygen concentrations. The potential use of pollen degradation as a proxy for all oxygen-sensitive degradation in either terrestrial or marine sediments may allow investigation of the role of oxygen-sensitive degradation over geological time. Pollen grains are often easily isolated, contain climate information that is useful in deducing environmental conditions at the time of deposition, and have varying degrees of susceptibility to oxidation^{17,18}. Thus estimations of oxygen exposure, via either morphological or chemical changes to pollen, can be directly and unambiguously compared to both radiocarbon dates²⁸ or stable carbon isotope compositions¹⁸, linking palaeoecology with global and oxygen cycles over much of the Phanerozoic era. □

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Subduction of Asian lithospheric mantle beneath Tibet inferred from models of continental collision

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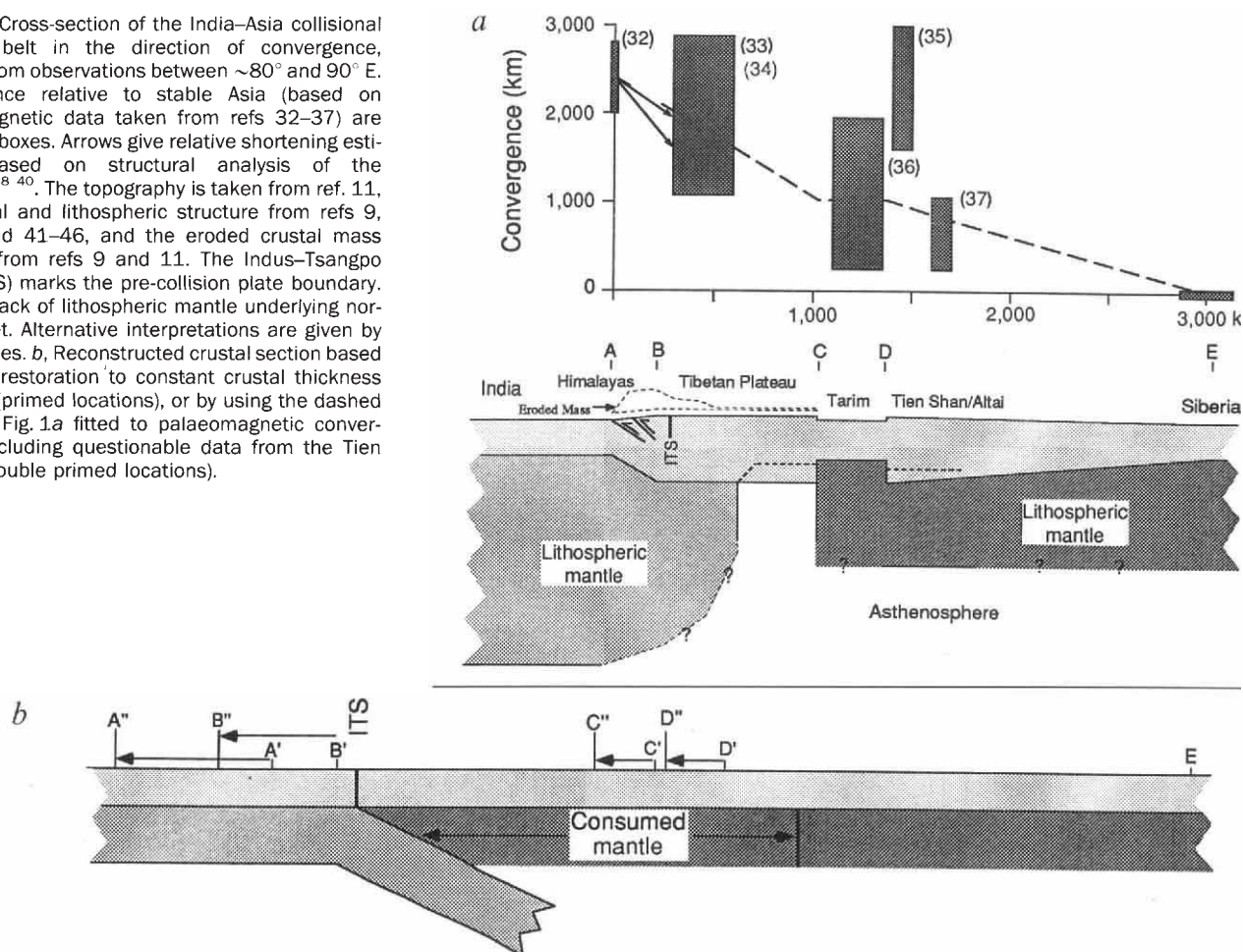
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THE relative northward motion of the Indian subcontinent that followed the onset of continental collision with Asia has produced extensive deformation of the Earth's crust, giving rise to the world's highest mountains in the Himalayan chain and the world's largest high-elevation region, the Tibetan plateau. The formation of the broad mountain belt implies that, contrary to the original tenets of plate tectonics, the lithospheric plates have experienced widespread deformation far from the plate boundary¹. Several models have been proposed²⁻⁶ to explain the manner in which this post-collisional deformation is distributed within the continental lithosphere of the Indian and Asian plates. Here we propose an alternative model in which subduction of the Asian lithospheric mantle develops following the collision of India. Our model is supported by numerical calculations of crustal deformation and thickening, and is consistent with available geological and geophysical data⁷⁻⁹. This picture suggests that lithospheric mantle is not deformed along with the crust, and would imply that continental collision zones are more analogous to oceanic subduction zones than was previously believed.

FIG. 1 *a*, Cross-section of the India-Asia collisional orogenic belt in the direction of convergence, inferred from observations between $\sim 80^\circ$ and 90° E. Convergence relative to stable Asia (based on palaeomagnetic data taken from refs 32-37) are shown as boxes. Arrows give relative shortening estimates based on structural analysis of the Himalaya^{38,40}. The topography is taken from ref. 11, the crustal and lithospheric structure from refs 9, 13-15 and 41-46, and the eroded crustal mass estimate from refs 9 and 11. The Indus-Tsangpo suture (ITS) marks the pre-collision plate boundary. Note the lack of lithospheric mantle underlying northern Tibet. Alternative interpretations are given by dashed lines. *b*, Reconstructed crustal section based either on restoration to constant crustal thickness of 38 km (primed locations), or by using the dashed line from Fig. 1*a* fitted to palaeomagnetic convergence, excluding questionable data from the Tien Shan³⁵ (double primed locations).

Although the India-Asia collision caused complex deformation of the upper crust with important three-dimensional effects, such as lateral extrusion by strike-slip faulting² and east-west extension¹⁰, the first-order effects of convergence can be appreciated by analysing a characteristic cross-section in the direction of convergence (Fig. 1). Estimates of crustal thickness together with palaeomagnetic data indicate that in-plane crustal thickening accounts for $\sim 80\%$ of the convergence^{11,12}. The remaining crustal mass, given by the distance from A' to A'' in Fig. 1, has been lost by erosion¹¹, laterally extruded into southeast Asia² and possibly absorbed by the mantle¹¹. Palaeomagnetic data, although limited in resolution, are consistent with structural estimates for shortening in the Himalayas and show that shortening is distributed throughout the Asian crust including regions well to the north of the Tibetan plateau such as the Tien Shan and Altai mountains (Fig. 1). Undeformed regions such as the Tarim and Qaidam basins behave as relatively rigid blocks in the deforming medium. In contrast to the crustal thickening, there is no evidence for corresponding thickening of the underlying lithospheric mantle. The homogeneous elevation of the Tibetan plateau belies the highly variable seismic velocity of the upper mantle⁷ with an anomalous zone of poor shear-wave transmission observed under the northern plateau. This region of poor shear-wave transmission has been defined by numerous studies^{7,13,14} and is spatially associated with Cenozoic volcanics, suggesting that the crust is underlain by anomalously hot mantle.

The upper-mantle structure provides an important constraint on models of the tectonic development of the India-Asia collision (Fig. 2*a-e*). Models have typically focused on the relative importance of underthrusting of the Indian plate beneath Tibet, distributed thickening of the Asian lithosphere and lateral extrusion of crustal material to the east². Lateral extrusion can be



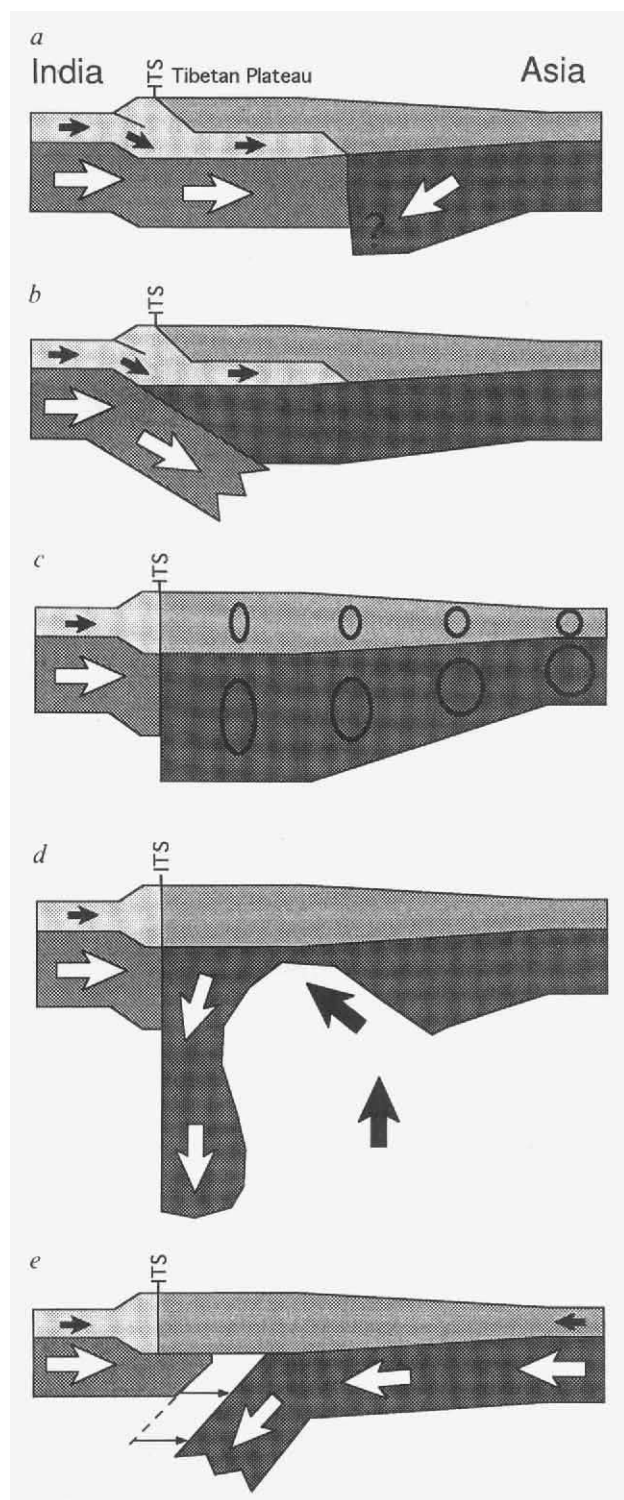


FIG. 2 Proposed models for the development of the India-Asia collision. *a*, Underthrusting of the Indian lithosphere under the Tibetan plateau^{4,5,47} following unspecified removal of Asian mantle. *b*, A variation of the underthrusting model in which the Indian lithosphere continues to subduct, but the crust is injected into the lower crust beneath the Tibetan plateau⁶. *c*, Distributed thickening of the Asian lithosphere by indentation of the stronger Indian lithosphere^{3,12,48}. *d*, Variation of *c*, including convective removal of the thickened lithospheric mantle¹⁷. This model is used to explain the inferred lack of lithospheric mantle beneath the northern Tibetan plateau. *e*, Subduction of Asian lithospheric mantle as investigated in this paper. Note proposal that retreat of the subducting mantle is responsible for upwelling mantle beneath the northern Tibetan plateau.

limited to ~20% of the total shortening^{11,12}. Underthrusting of India can also be eliminated as the primary mechanism of shortening by its inconsistency with palaeomagnetic data (Fig. 1), the lack of Indian shield-type lithosphere under northern Tibet¹⁵ and by estimates of the present rate of convergence between India and southern Tibet¹⁶. These data are consistent and imply that underthrusting of India is limited to, at most, the southern Tibetan plateau, leaving distributed lithospheric thickening (Fig. 2c) as the most widely accepted model. Quantitative models of distributed thickening have, however, included the primary assumption that the lithospheric mantle thickens together with the overlying crust^{3,17}. To be consistent with the observed upper-mantle structure, it has been proposed that this thickened lithospheric mantle is removed by flow of the convectively unstable lithosphere (Fig. 2d)¹⁷.

Subduction of the Asian lithospheric mantle (Fig. 2e) provides an alternative model that has received only limited consideration. Some component of mantle subduction has been proposed to explain the basic volcanism of the northern plateau^{18,19} and the structure of the Altai mountains to the north²⁰, but these models have generally made Asian mantle subduction subsidiary to the subduction of Indian lithosphere. But recent models for collisional tectonics have emphasized the decoupling of the crust and mantle^{21,22}, and these models lead to the conclusion that the large-scale structure and deformation of the orogen are consistent with Asian mantle subduction being the dominant mode of deformation, as discussed below.

Numerical models^{21,23} have allowed more quantitative assessment of the styles of deformation associated with intra-continental mantle subduction, and have been successful in explaining many features of collisional orogens, particularly in small orogens with limited (tens of kilometres) convergence^{24,25}. A similar model calculation with large convergence²⁶ (Fig. 3) predicts that intra-continental mantle subduction results in a distinctly asymmetric style of crustal deformation with diffuse deformation in the crust overlying the subducting plate (pro-wedge)²¹, and more localized deformation in the crust overlying the overriding mantle plate (retro-wedge)²¹. The crustal suture marking the point of initiation of subduction migrates continuously in the direction of subduction (Fig. 3a-e), resulting in an orogen primarily composed of crust derived from the subducting plate. The asymmetry of strain and mass distribution with respect to the crustal suture are general results of mantle subduction, and are not strongly dependent on specific model parameters. The existence of the high-elevation plateau requires only that temperature be high enough to allow viscous flow in the lower crust, a condition easily met for crust of 70 km thickness.

An important variation to this model is produced by retreat of the mantle slab (Fig. 3f) during convergence and subduction. Retreat is the process of slab sinking that leads to trench migration towards the ocean and back-arc spreading in oceanic subduction settings²⁷. In an intra-continental setting, roll-back creates space between the opposing mantle lithospheric plates, resulting in upwelling of asthenospheric mantle (potentially to the base of the crust), thus providing a mechanism for the emplacement of hot upper-mantle beneath an orogenic plateau. Retreat also modifies the structure of the orogen, changing the transported position of the crustal suture relative to the deformational fronts of the orogen, thereby reducing the backthrusting and mass of the retro-wedge.

These numerical models, although not intended as a complete simulation of the India-Asia collisional system, can be used to interpret many of the features of the system. Although before collision the Indian plate was probably subducting down to the north⁸, the models clearly suggest that following collision the polarity of subduction switched, a process observed in many locations at present²⁸. Subsequently, Asian mantle has subducted to the south (Fig. 4). This interpretation is supported by four observations. First, the overall morphology of the orogenic belt is consistent with this model, with the Tibetan plateau rep-

FIG. 3 Finite-element model for deformation of a 40-km-thick crust overlying a continental mantle subduction zone. Boundary conditions are imposed at the base of the crust and correspond to lithospheric mantle to the right of the original suture position moving from right to left at a constant velocity (large white arrows), whereas mantle to the left of this position remains stationary. Note that the lithospheric mantle is not modelled, and the model makes no prediction of the ultimate fate of the subducted mantle. The crust deforms according to a Coulomb plastic yield criterion at low temperature and in a viscous fashion at higher temperatures. Temperature is calculated assuming one-dimensional steady-state heat transfer. The thickened crust is flexurally compensated on an elastic plate. The deformed mesh is a passive material tracking grid, not the finite element mesh which is not shown. The displacement of the original crustal suture is indicated by the small black arrow. Details of the model and numerical method are given in refs 21, 23, 24. *a*, Initial condition. ('No VE', no vertical exaggeration.) *b*, Deformation after $\Delta x = 75$ km of convergence. Asymmetry in the deformation develops immediately with localized deformation to the downstream or retro-²¹ side of the orogen, and diffuse deformation on the upstream or pro-²¹ side. *c*, Deformation after 150 km of convergence. *d*, Deformation after 225 km of convergence. A high-elevation plateau develops due to increased temperature that weakens the base of the thickened crust. *e*, Deformation after 1,000 km of convergence. A large plateau develops, with the original crustal suture located approximately one-third of the distance across the plateau from the retro-deformation front. *f*, A second model after 1,000 km of convergence, with retreat of the subducting slab at half the rate of subduction. Retreat is defined as the migration of the mantle detachment point from left to right, and creates space for asthenospheric upwelling. Models can be compared to the India-Asia orogen with subduction of Indian or Asian mantle by considering left to be north or south, respectively. However, the location of the crustal suture near the plateau edge virtually eliminates subduction of India as a viable model.

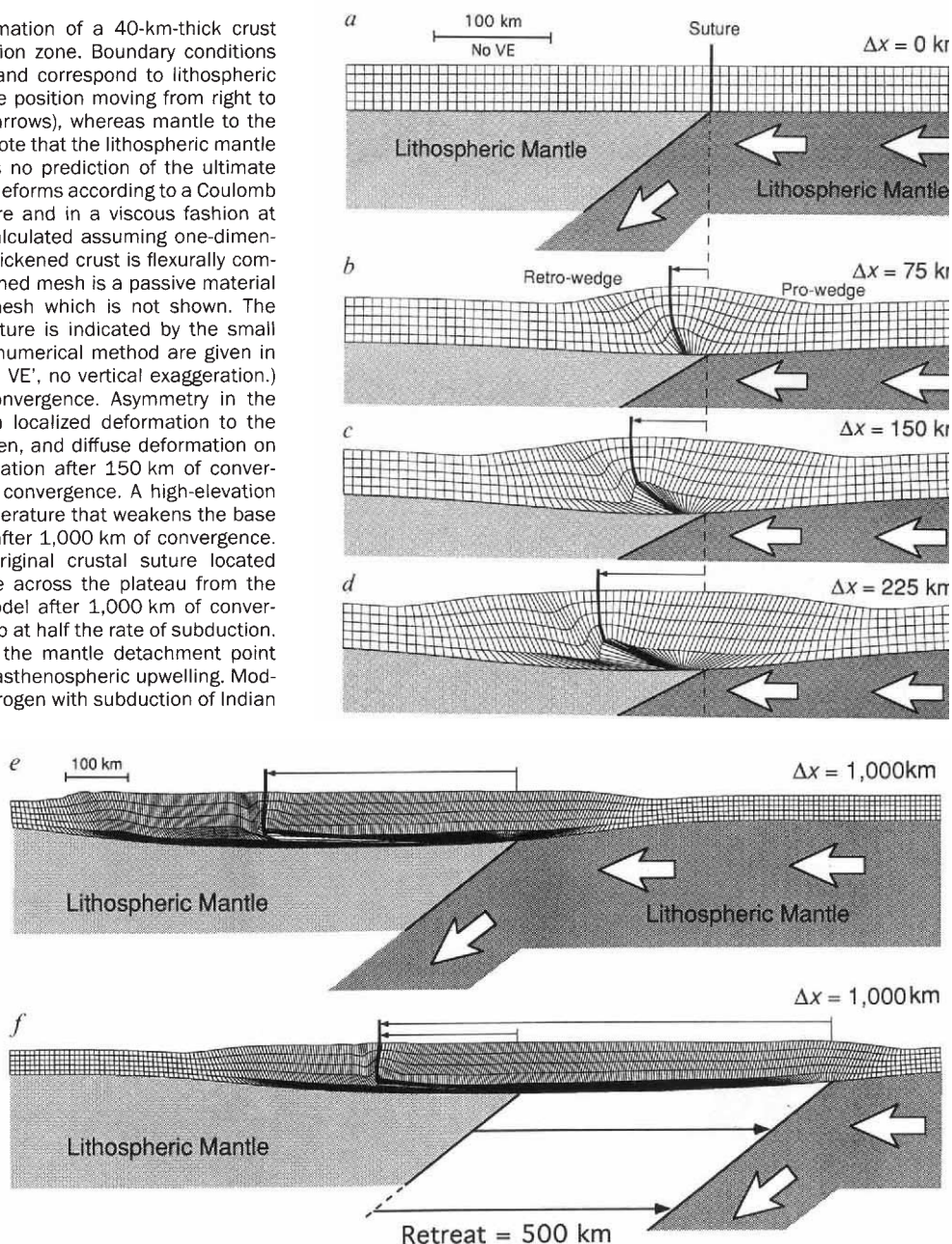
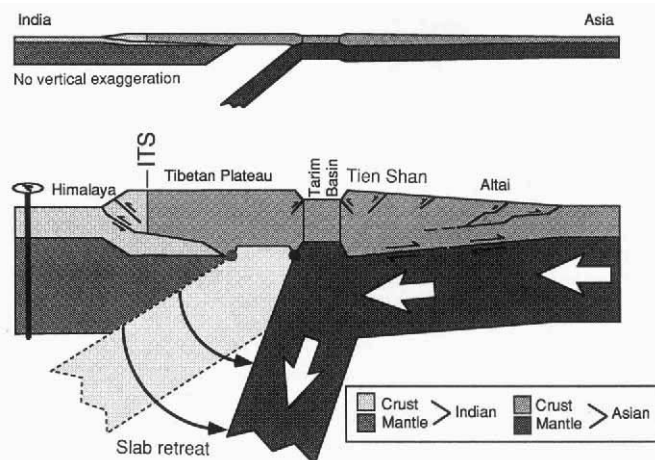


FIG. 4 Interpreted structure, with and without vertical exaggeration, of the India-Asia collision based on numerical models. The model is shown in a frame of reference with India fixed, but an equivalent interpretation is that India moves north, parting Asian crust and mantle, allowing the negatively buoyant mantle to subduct. Asian mantle subducts beneath the Tibetan plateau and retreats to a present-day position beneath the Kunlun mountains on the northern margin of the plateau, thereby opening an asthenospheric window beneath the northern plateau. The Indian crust is shortened along a mid- to lower-crustal detachment to form the retro-wedge of localized backthrusting in the Himalayas. North of the Kunlun, including the rigid blocks of the Tarim and Qaidam basins, is a broad zone forming a pro-wedge of diffuse deformation and shortening that extends to the Siberian shield.



resenting the high-elevation plateau overlying a weak, viscous, lower crust bounded by deforming crustal wedges to the north and south. Second, the distribution of deformed crust relative to the Indus–Tsangpo Suture is consistent with Asian mantle subduction; the suture is located <300 km from the southern edge of deformation, and between 2,000 and 2,500 km from the northern edge. Even when the mass lost by denudation in the Himalaya is included, the asymmetry in mass distribution suggests significant loss of Asian mantle by subduction. Third, the localization of crustal shortening and strain is consistent with a model of Asian mantle subduction. Shortening in the Himalaya has been localized on individual thrust systems such as the Main Central Thrust²⁹, consistent with the localized deformation of the model retro-wedge. This localization effect is intensified by exhumation²¹, as has occurred in the Himalaya. In contrast, even though the bulk of the convergence has resulted in thickening of the Asian crust, this strain has not produced similar localized thrust systems, but results in diffuse deformation of the crust. In the context of the model, the broad zone of diffuse deformation from the Tien Shan and Altai north to the Siberian shield constitutes a critical pro-wedge³⁰ maintaining its taper by slip along one or more crustal detachments above the subducting Asian mantle. Fourth, by introducing retreat of the subducting mantle, upwelling hot mantle produces the anomalous seismic structure of the northern Tibetan plateau. Partial melting of the subducting Asian mantle below this hot mantle material is consistent with the volcanism in the northern plateau^{19,31}.

With respect to mantle deformation, these mantle subduction models represent the alternative end member to the distributed thickening model (Fig. 2c) which includes the assumption that crust and lithosphere deform as a single layer. That both end-member models are, with the addition of secondary or consequent processes, consistent with orogen-scale observations from the India–Asia collisional orogen means that either one (and probably intermediate models) must be considered as valid explanations. Future observations should be designed to distinguish between them.

The applicability of the mantle subduction model to the India–Asia collision has general implications. The model is consistent with our understanding of the deformation of the Earth's mantle at oceanic subduction zones and in small collisional orogens, where no pervasive lithospheric mantle thickening has been observed. If the model also applies at the scale of the India–Asia collision, this suggests that the broad zones of deformation at continental collision boundaries¹ are confined to the crust, that lithospheric mantle is not readily deformed and that (consistent with the ideas of plate tectonics), subduction, albeit mantle subduction, is the principal expression of lithospheric convergence. □

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Implications of early hominid labyrinthine morphology for evolution of human bipedal locomotion

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THE upright posture and obligatory bipedalism of modern humans are unique among living primates. The evolutionary history of this behaviour has traditionally been pursued by functional analysis of the postcranial skeleton and the preserved footprint trails of fossil hominids. Here we report a systematic attempt to reconstruct the locomotor behaviour of early hominids by looking at a major component of the mechanism for the unconscious perception of movement, namely by examining the vestibular system of living primates and early hominids. High-resolution computed tomography was used to generate cross-sectional images of the bony labyrinth. Among the fossil hominids the earliest species to demonstrate the modern human morphology is *Homo erectus*. In contrast, the semi-circular canal dimensions in crania from southern Africa attributed to *Australopithecus* and *Paranthropus* resemble those of the extant great apes. Among early *Homo* specimens, the canal dimensions of Stw 53 are unlike those seen in any of the hominids or great apes, whereas those of SK 847 are modern-human-like.

Functional analyses of hominid postcranial fossils and preserved footprint trails have prompted conflicting interpretations, characterizing the early stages of hominid locomotion either as a modern human-like obligatory bipedalism^{1,2} or as a more primitive behaviour which combined arboreal climbing with a bipedal gait unlike that seen in modern humans^{3,4}. We have explored the potential of a new approach to the study of early hominid locomotion by focusing on the vestibular apparatus, part of the system of receptors which monitors movements. Modern human locomotor behaviour makes particular demands on the vestibular apparatus for it involves the maintenance of an upright body posture by balancing on very small areas of support. The morphology of the vestibular apparatus that is preserved in fossils comprises the three bony semicircular canals, and their

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dimensions reflect the arc size of the enclosed membranous semicircular ducts. Theoretical and experimental neurophysiological evidence indicates a direct relationship between duct size and the sensitivity and time constants of the semicircular canal monitoring system⁵⁻⁹, and studies of birds and prosimians have suggested that canal size and locomotor behaviour are interrelated¹⁰⁻¹².

Methods for visualization of the bony labyrinth in fossil hominid crania using high-resolution computed tomography (CT) have been reported^{13,14}. The sample, comprising 31 extant primate species and 12 hominid fossils, is listed in Table 1. Height and width measurements of the arc of each semicircular canal were taken from CT scans, and these were used to calculate the radius of curvature (R) of the arc (Fig. 1), which is given in Table 2 for the great ape and hominid species.

In the extant primate sample, the arc size of each of the three semicircular canals is correlated with body mass (Fig. 2). Taking body mass into account, modern humans have larger anterior

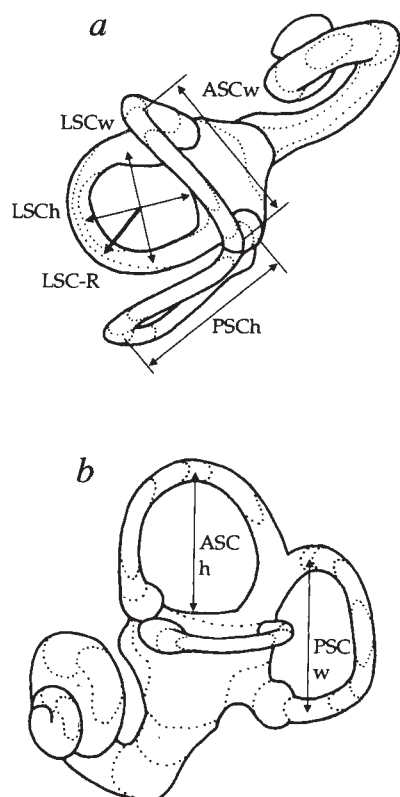


FIG. 1 The superior (a) and lateral (b) aspects of a left human bony labyrinth, reconstructed from transverse and sagittal CT scans of the temporal bone region respectively. Labyrinth outlines that appear in individual scans are shown as dotted lines. The method used to obtain these CT scans is described elsewhere^{13,14,30}. The height (h) and width (w) of the anterior (ASC), posterior (PSC) and lateral (LSC) semicircular canals were taken, using landmarks on the wall of the vestibule and in the centre of the canals' lumina (ASCw, PSCw, LSCw and LSCr in transverse CT scans, and ASCh and PSCw in sagittal scans). Experimentally, these dimensions can be taken from CT scans with a maximum error of ± 0.1 mm³⁰. The radius of curvature (R) of each canal, listed in Table 2 and plotted in Fig. 2, is defined as half the average of the canal height and width ($0.5(h+w)/2$), and is indicated for the lateral canal here (LSC-R). The membranous semicircular ducts seem to be consistently located along the outer wall of the bony canals^{31,34,35}, and dimensions defined using landmarks on the outer canal wall may therefore give better estimates of the functionally important duct sizes. Such landmarks were not employed here, however, as they result in less accurate measurements than landmarks in the centre of the canal's lumen when taken from CT scans³⁰.

TABLE 1 Number of specimens for the extant species

	CT	Published	Code
Extant species			
<i>Homo sapiens</i>	53	20 (ref. 31)	Hs
<i>Pan troglodytes</i>	7	1 (ref. 32)	Pt
<i>Pan paniscus</i>	6	—	Pp
<i>Gorilla gorilla</i>	6	1 (ref. 32)	Gg
<i>Pongo pygmaeus</i>	7	1 (ref. 32)	Po
<i>Hylobates symphalangus</i>	2	—	
<i>Hylobates moloch</i>	1	—	
<i>Hylobates pileatus</i>	1	—	
<i>Macaca fascicularis</i>	3	—	
<i>Macaca mulatta</i>	—	15 (ref. 33)	
<i>Macaca nemestrina</i>	—	1 (ref. 32)	
<i>Nasalis larvatus</i>	1	—	
<i>Cercopithecus aethiops</i>	—	1 (ref. 34)	
<i>Cercopithecus nictitans</i>	—	1 (ref. 34)	
<i>Papio cynocephalus</i>	—	1 (ref. 34)	
<i>Papio hamadryas</i>	—	1 (ref. 32)	
<i>Papio ursinus</i>	1	—	
<i>Theropithecus gelada</i>	1	—	
<i>Mandrillus sphinx</i>	1	—	
<i>Lagothrix lagothricha</i>	1	—	
<i>Alouatta seniculus</i>	1	1 (ref. 32)	
<i>Saimiri sciureus</i>	1	17 (refs 33, 35)	
<i>Cebus sp.</i>	—	1 (ref. 35)	
<i>Aotus trivirgatus</i>	—	1 (ref. 35)	
<i>Callithrix jacchus</i>	—	1 (ref. 34)	
<i>Tarsius bancanus</i>	—	1 (ref. 12)	
<i>Lemur macaco</i>	—	1 (ref. 32)	
<i>Lemur mongoz</i>	—	1 (ref. 34)	
<i>Nycticebus coucang</i>	—	2 (refs 12, 34)	
<i>Propithecus diadema</i>	1	—	
<i>Indri indri</i>	1	—	
Fossil hominids			
<i>Homo erectus</i>	OH9, Sangiran 2, Sangiran 4		He
Early <i>Homo</i>	Stw 53g (d), SK 847		53
<i>Australopithecus africanus</i>	Taung, Sts 5, Sts 19, MLD 37/38		Aa
<i>Paranthropus robustus</i>	SK 46, SK 47, SK 879		Pr

Measurements were taken from CT scans and from the literature. Where both sources provide canal dimensions for the same species, these correspond well³⁰. For the great ape and hominid species, the codes used in Fig. 2 are given.

and posterior canals and a smaller lateral canal than the great apes (Fig. 2). These differences are also clearly demonstrated by the relative canal sizes listed in Table 2. Among the fossil hominids, the australopithecines show great-ape-like proportions and *H. erectus* shows modern-human-like proportions. The specimen Stw 53, provisionally attributed to *H. habilis*, differs from all other hominids by having a particularly large lateral semicircular canal, and this morphology shows greatest similarity to the pattern observed for large cercopithecoids. These unexpected canal proportions were noted for the perfectly preserved, air-filled, left labyrinth in Stw 53g. The right petrous pyramid of the same individual, Stw 53d, is internally fragmented and the only two measurements (ASCw, PSCw) that could be taken are identical to those on the left side. The specimen SK 847 has not been included in Fig. 2 because no realistic body mass estimates are yet available. However, its absolute and relative canal dimensions show closest resemblance to modern humans (Table 2). It is worth mentioning that the planar orientations of the semicircular canals in the cranium (the anterior and posterior ones relative to the midsagittal plane, and the lateral one relative to the basicranial orientation basion-nasion) are not significantly different in modern humans, the African great apes and the fossil hominids.

TABLE 2 Absolute and relative radii of curvatures of the anterior, posterior and lateral semicircular canals of the great apes and hominids

	Absolute radius of curvature						Relative radius of curvature		
	ASC-R		PSC-R		LSC-R		ASC	PSC	LSC
	Mean	s.d.	Mean	s.d.	Mean	s.d.			
<i>Homo sapiens</i>	3.2	0.24	3.1	0.31	2.3	0.44	37	36	26
<i>Pan troglodytes</i>	2.7	0.20	2.8	0.25	2.5	0.25	34	35	31
<i>Pan paniscus</i>	2.6	0.19	2.5	0.16	2.4	0.18	35	34	32
<i>Gorilla gorilla</i>	2.9	0.15	3.0	0.28	3.0	0.25	32	34	34
<i>Pongo pygmaeus</i>	2.7	0.25	2.5	0.21	2.4	0.11	35	33	31
<i>Homo erectus</i>	3.2	0.06	3.1	0.25	2.1	0.23	38	37	25
SK 847	3.1	—	2.9	—	2.3	—	37	35	28
Stw 53g	2.9	—	2.8	—	2.8	—	34	33	33
<i>Australopithecus africanus</i>	2.4	0.26	2.6	0.19	2.2	0.17	34	36	30
<i>Paranthropus robustus</i>	2.6	0.12	2.7	0.26	2.5	0.12	34	34	32

The absolute radius is given in millimeters (mean and standard deviation listed for species), and the relative radius in per cent (sum of the three canals is 100%). ASC, PSC and LSC represent anterior, posterior and lateral semicircular canals, respectively.

As all four great ape species have similarly proportioned semicircular canals, it seems likely that this pattern represents the ancestral condition of the hominids. This is supported by the fact that the oldest fossil hominids thus far investigated show the great-ape condition. Hence, the evolutionary history of the human labyrinth is characterized by the subsequent enlargement of the anterior and posterior semicircular canals and reduction of the lateral canal. The exact functional consequences of enlarging the arc of a semicircular duct and canal are not fully understood, because of uncertainty over some fundamental aspects, such as the nature of cupula movement in the ampulla¹⁵. How-

ever, models of endolymph movement as well as the results of neurophysiological experiments indicate a link between a larger arc size and an increase in sensitivity⁵⁻⁹. Increased sensitivity of the vertically oriented anterior and posterior canals in humans would make sense because the role of the vestibular system in coordinating upright bipedal behaviour through the vestibular reflexes particularly involves monitoring body movements in the vertical plane.

Among the fossil hominids investigated, the earliest species to demonstrate the modern human semicircular canal morphology is *Homo erectus*. Hence, if the enlargement of the anterior and

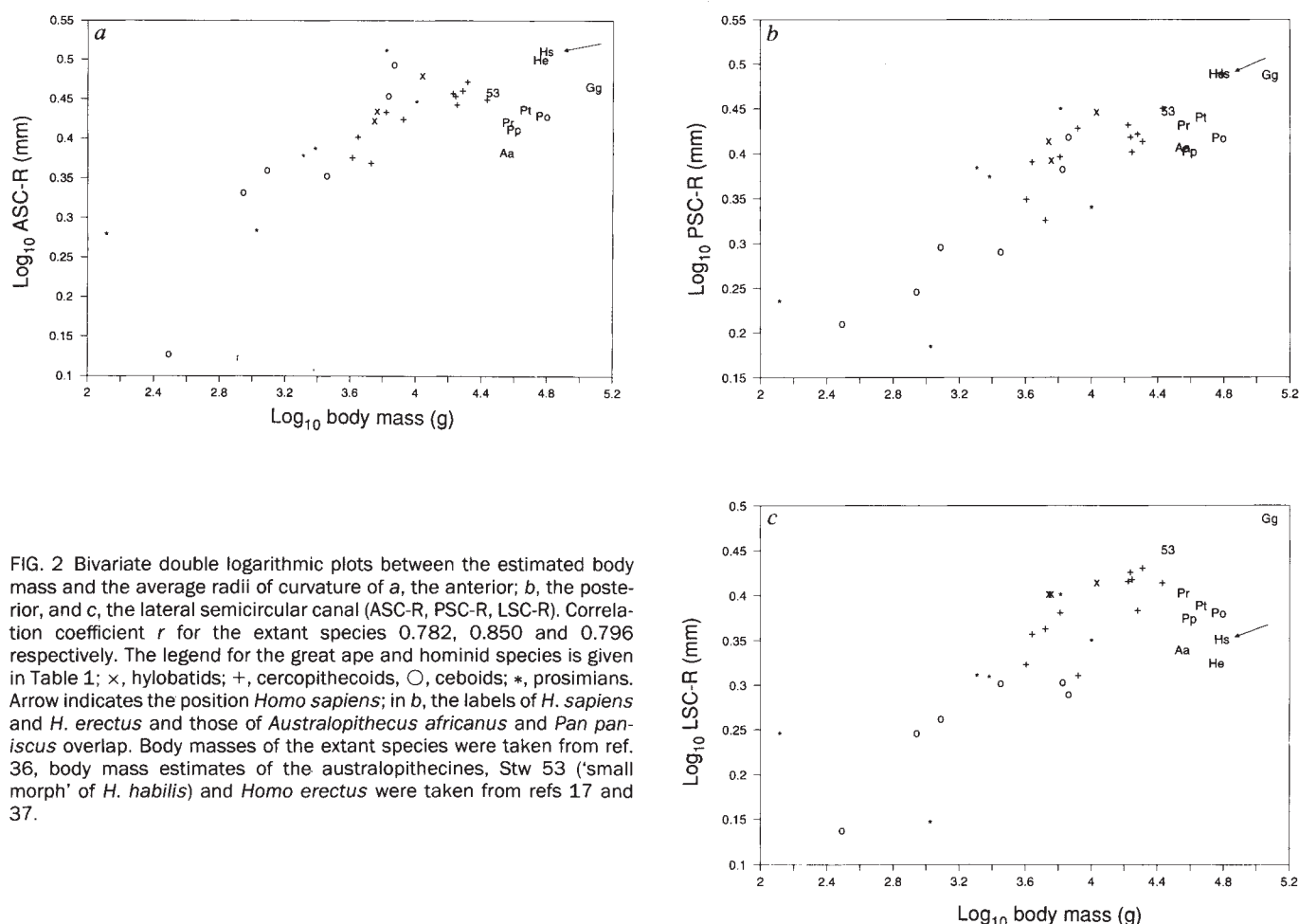


FIG. 2 Bivariate double logarithmic plots between the estimated body mass and the average radii of curvature of a, the anterior; b, the posterior, and c, the lateral semicircular canal (ASC-R, PSC-R, LSC-R). Correlation coefficient r for the extant species 0.782, 0.850 and 0.796 respectively. The legend for the great ape and hominid species is given in Table 1; x, hylobatids; +, cercopithecoids, O, ceboids; *, prosimians. Arrow indicates the position *Homo sapiens*; in b, the labels of *H. sapiens* and *H. erectus* and those of *Australopithecus africanus* and *Pan paniscus* overlap. Body masses of the extant species were taken from ref. 36, body mass estimates of the australopithecines, Stw 53 ('small morph' of *H. habilis*) and *Homo erectus* were taken from refs 17 and 37.

posterior canals is functionally related to modern human-like obligatory bipedalism, then at least in this respect the vestibular apparatus of the australopithecines was not adapted to this type of locomotor behaviour. These observations support studies of the postcranial fossil record which have concluded that *H. erectus* was an obligatory biped^{16,17}, whereas *A. africanus* showed a locomotor repertoire comprising facultative bipedalism as well as arboreal climbing^{18–20}. The labyrinthine evidence is consistent with proposals that bipedalism in australopithecines was characterized by a substantial postural component^{21,22} and by the absence of more complex movements such as running and jumping³. Such behaviour will predominantly make functional demands on the utricular and saccular part of the vestibular apparatus, and is therefore not clearly reflected in the semicircular canal morphology. It is not evident from the semicircular canal proportions that *P. robustus* had a less arboreal locomotor repertoire than *A. africanus*, as has been concluded from their postcranial morphology²³.

The specimen Stw 53, provisionally referred to *H. habilis*, has semicircular canal proportions not seen in any of the other fossil or extant hominids or great apes. Functional interpretation of this morphology can only be speculative, but the similarity with the canal proportions in large cercopithecoids suggests that Stw 53 relied less on bipedal behaviour than the australopithecines. Interestingly, similar observations were reached from an analysis of the postcranial bones of OH 62 (ref. 4), a specimen that has been assigned to the same species as Stw 53 on the basis of similarities in their maxillary and dental morphology²⁴. Phylogenetically, the unique labyrinth of Stw 53 represents an unlikely intermediate between the morphologies seen in the australopithecines and *H. erectus*.

The specimen SK 847 has both been associated with *H. erectus*^{25,26} and *H. habilis*, in particular with Stw 53 (ref. 27). The modern-human-like labyrinth of SK 847 is consistent with the attribution to *H. erectus*, and the extreme differences in labyrinthine morphology between SK 847 and Stw 53 make attribution of both specimens to the same species, on this evidence alone, highly unlikely. The specimen Sts 19 is part of the conventional *A. africanus* hypodigm, but has also been considered as a basicranium of early *Homo*²⁸. As the labyrinth of Sts 19 is very similar to that in the other three *A. africanus* specimens, and major aspects of its overall morphology, such as petrous pyramid orientation and basicranial flexion, can easily be accommodated in normal species variation^{29,30}, the conventional attribution is followed here. However, omission of Sts 19 from the *A. africanus* hypodigm would not change our conclusions.

This study demonstrates that the morphology of the bony labyrinth has the potential to provide information about both the locomotor behaviour and the phylogenetic relationships of early hominids. Although the labyrinth can only be accessed in fossils by CT scanning, it has the advantage that its morphology can be studied in juvenile specimens because it attains its adult shape and size long before birth. Moreover, relatively large samples are available because petrous pyramids containing an intact labyrinth are common in fossil hominid assemblages. □

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Cytotoxic T-cell memory without antigen

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MEMORY is a hallmark of the immune system and ever since its recognition there has been considerable interest in understanding how immunity is maintained^{1–5}. The current model is that long-term memory is dependent on persistent antigenic stimulation^{6–11}. We report here results that challenge this view and provide evidence that antigen is not essential for the maintenance of CD8⁺ T-cell memory. We show that memory CD8⁺ cytotoxic T lymphocytes persist indefinitely in the absence of priming antigen, retain the memory phenotype (CD44^{hi}), and provide protection against virus challenge. These findings suggest a re-evaluation of our current thinking on mechanisms involved in maintaining immunity and have implications towards designing effective vaccination strategies.

Adult mice that have undergone an acute lymphocytic choriomeningitis virus (LCMV) infection exhibit life-long cytotoxic T lymphocyte (CTL) memory (Fig. 1). How is this memory maintained? Although the infection is resolved within 2 weeks, it is possible that low levels of antigen may persist in specialized depots such as follicular dendritic cells (FDC)¹². Thus, using the originally infected mice, it is difficult experimentally to evaluate the true role of antigen in maintenance of memory. To overcome this problem, purified CD8⁺ T cells devoid of antigen-presenting cells were isolated from LCMV immune mice and their survival monitored after adoptive transfer into uninfected mice. Before adoptive transfer, these fluorescence-activated cell sorted (FACS)-purified CD8⁺ T cells (>99.9% pure) were rigorously checked for the presence of viral material. No infectious virus was detectable either by plaque assays or mouse infectivity

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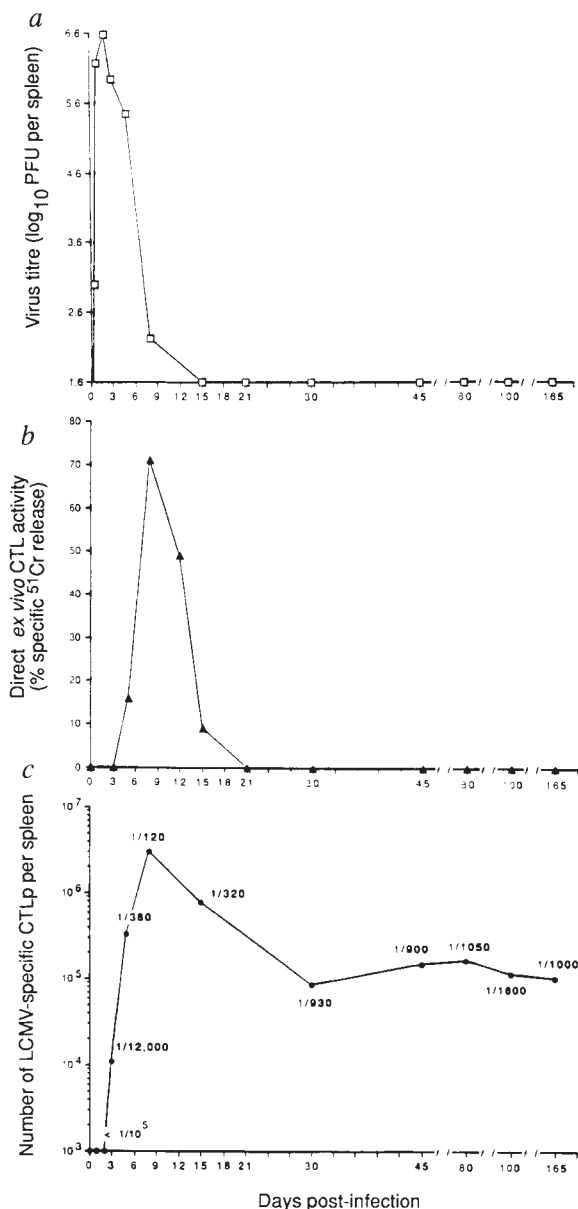


FIG. 1 Long-term CTL memory following acute LCMV infection. Adult B6.PL-Thy1^a (H-2^b, Thy-1.1) mice were injected intraperitoneally with 2×10^5 PFU of LCMV Armstrong strain. At indicated times post-infection, they were monitored for virus levels in the spleen (a), and their virus-specific CTL responses were analysed by checking for direct ex vivo cytotoxicity on syngeneic LCMV-infected targets (b), and also by quantifying the number of LCMV-specific CTL precursors (CTLp) by limiting dilution assays (c). LCMV titres in the spleen were determined by plaque assay as described¹⁸. Direct primary ex vivo spleen CTL activity was measured in standard 6-h ⁵¹Cr release assays¹⁸. The per cent cytotoxicity on LCMV-infected MC57 (H-2^b) fibroblast cell line targets is shown at an effector:target (E:T) ratio of 50:1 (per cent cytotoxicity on uninfected targets at the same E:T ratio has been subtracted). For the limiting dilution assay, spleen cells from immunized mice were cultured in graded doses (12–24 wells per dose) with 8×10^5 syngeneic spleen cell feeders (1,200 rad) from uninfected mice and 2×10^5 syngeneic stimulator splenocytes (1,200 rad) from LCMV-carrier mice in 96-well flat-bottom plates. Recombinant human interleukin-2 from *Escherichia coli* (specific activity 18×10^6 IU mg⁻¹; Cetus Corp.) was added at 50 U ml⁻¹ final concentration. After 8 days, the contents from each well were split to test CTL activity against LCMV-infected and uninfected MC57 targets in 6-h ⁵¹Cr-release assays. LCMV-specific CTLp frequencies²¹ are displayed above the absolute numbers of specific CTLp plotted in the figure. The data shown are the averages of 2–6 mice at each timepoint.

<twofold drop over a 1-year period (data not shown). A host-derived (Thy 1.2) LCMV-specific CTL response was not detected at any time point. Note that under the *in vitro* restimulation conditions used, virgin T cells do not generate an antiviral CTL response. In the next series of experiments, we did limiting dilution analysis to determine the number of LCMV-specific CTL precursors (CTLp) in the input CD8⁺ population and in recipient mice at various times post-transfer. This allows for a more precise and quantitative assessment of T-cell memory. The data shown in Fig. 2a document the remarkable stability of memory CTL in an environment free of specific antigen. Twenty thousand LCMV-specific CTLp were transferred into uninfected mice and there was minimal to no drop in their frequency or numbers over a 26-month period. The transferred CTL were present not only in the spleen but were also found at comparable frequencies in the blood and lymph nodes. These results demonstrate that memory CTL are not a sessile population within the spleen but are continuously circulating, a property befitting a cell needed for immune surveillance.

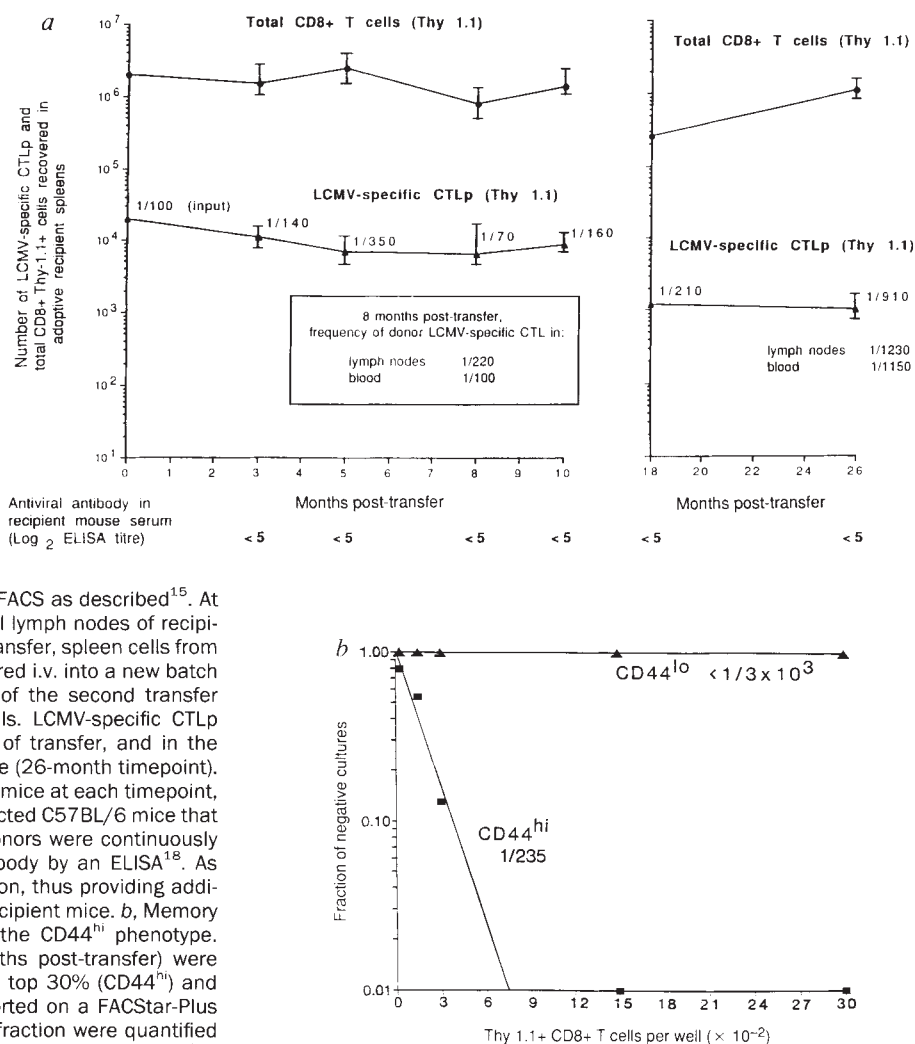
Memory T cells are characterized by higher levels of adhesion molecules such as CD44^{16,17}. To determine whether the CD44^{hi} phenotype was retained in the absence of antigen, spleen cells from adoptive recipients (5 months post-transfer) were sorted by FACS into CD44^{hi} and CD44^{lo} populations, and the frequency of LCMV-specific CTLp in each fraction determined by limiting dilution analysis. All the CTL activity segregated with the CD44^{hi} population (Fig. 2b). Thus, the results of Fig. 2a and b show unequivocally that LCMV-specific CTL persist indefinitely and retain the CD44^{hi} memory phenotype in the absence of priming antigen. To rule out accidental exposure of the recipient mice to LCMV, we routinely monitored them for seroconversion or priming of their T cells, and checked their spleens for viral RNA by PCR. All tests were negative. In addition, to check for the possibility of a 'latent' or low-level LCMV infection, two recipient mice that received 1×10^6 sorted CD8⁺ T cells from LCMV-immune mice 12 months earlier were immunosuppressed by whole-body irradiation (650 rad) and examined for the emergence of virus. Two weeks after irradiation, these mice were killed and various tissues (blood, spleen, lymph nodes, liver, lung, brain and kidney) analysed for the presence of virus; no infectious virus was detected.

We next investigated whether memory CTL showing long-term survival in an antigen-free environment can provide protection against reinfection. A virulent strain of LCMV (clone 13) that causes a persistent infection in naive adult mice but is cleared by immune mice was used as the challenge virus¹⁸. Ability

assays (<1 per 5×10^6 cells). The *in vivo* infectivity assays were also negative on injecting mice with disrupted (sonicated or anti-CD8 + C'-treated) cells. This additional control rules out the possibility that contaminating virus in the CD8⁺ T-cell population was being kept in check by antiviral CTL. A quantitative polymerase chain reaction (PCR), using primers amplifying LCMV sequences encoding the major CTL epitopes¹³, failed to reveal any viral genetic material (<1 genome equivalent per 10^5 CD8⁺ T cells). All immunohistochemical tests for viral antigen were also negative. In addition, these sorted CD8⁺ T cells were unable to stimulate LCMV-specific CTL clones *in vitro* or prime host T cells *in vivo*. It should also be noted that the LCMV strain used in this study does not infect CD8⁺ T cells¹⁴.

FACS-purified, virus-free CD8⁺ T cells from LCMV-immune mice (>90 days after infection) were transferred into uninfected mice and at various times post-transfer, recipient mice were killed and their spleen cells restimulated *in vitro* to check for LCMV-specific CTL activity. To distinguish the transferred T cells from recipient host T cells, mice congenic at the Thy 1 locus were used¹⁵. The identity of the effector cells was determined by treatment with anti-Thy 1.1 or anti-Thy 1.2 plus C'. Donor-derived (Thy 1.1) CTL responses were recovered at all time points tested and, based on lytic units per spleen, showed

FIG. 2 CTL memory does not wane in the absence of antigen. **a**, Quantification of virus-specific CTLp and total donor CD8⁺ T cells after transfer into uninfected mice. 2×10^6 FACS-sorted CD8⁺ T cells (>99.9% pure) from LCMV-immune B6.PL-Thy1^a mice (>90 days post-infection) were injected intravenously (i.v.) into uninfected, congenic C57BL/6 (H-2^b, Thy-1.2) mice (irradiated 1 day before transfer, 650 rad). The input CD8⁺ T-cell population contained 2×10^4 LCMV-specific CTLp. Before adoptive transfer these purified CD8⁺ T cells were tested for contaminating virus by highly sensitive immunological, viral, and molecular assays, and were shown to be free of any detectable LCMV genome or antigen (see text). At indicated times post-transfer, the recovery of LCMV-specific CTLp from recipient spleens was quantified by limiting dilution assays, and total donor and host CD8⁺ T cells were analysed by FACS as described¹⁵. At the 8-month timepoint the blood and peripheral lymph nodes of recipient mice were also analysed. 18 months post-transfer, spleen cells from the remaining adoptive recipients were transferred i.v. into a new batch of uninfected C57BL/6 mice (650 rad). Each of the second transfer recipients received 2×10^5 Thy-1.1⁺ CD8⁺ cells. LCMV-specific CTLp were quantified in the input cells at the time of transfer, and in the spleen, lymph nodes and blood of recipient mice (26-month timepoint). The data shown represent the average of 2 to 3 mice at each timepoint, with vertical bars indicating the range. All uninfected C57BL/6 mice that received purified CD8⁺ T cells from immune donors were continuously monitored for the presence of anti-LCMV antibody by an ELISA¹⁸. As shown in this figure, there was no seroconversion, thus providing additional evidence for absence of viral antigen in recipient mice. **b**, Memory T cells transferred to uninfected mice retain the CD44^{hi} phenotype. Spleen cells from adoptive recipients (5 months post-transfer) were stained with FITC-anti-CD44 (Pharmingen). The top 30% (CD44^{hi}) and bottom 30% (CD44^{lo}) fluorescent cells were sorted on a FACStar-Plus (Becton-Dickinson). LCMV-specific CTL in each fraction were quantified in limiting dilution assays. Donor (Thy-1.1) and host (Thy-1.2) CD8⁺ T cells in each fraction were determined by FACS.



to control clone 13 infection is a definitive *in vivo* marker for CTL memory. Three groups of uninfected mice that had received $1-2 \times 10^6$ sorted CD8⁺ T cells from LCMV-immune mice were used in these experiments: (1) CD8⁺ T cells from immune Thy 1.1 mice into uninfected Thy 1.2 recipients; (2) CD8⁺ T cells from immune Thy 1.1 mice into uninfected Thy 1.1 recipients; and (3) CD8⁺ T cells from immune BALB/c mice into severe combined immunodeficient (SCID) mice. When challenged with LCMV clone 13, all of these mice were able to control the infection. In contrast, normal immunocompetent adult mice infected with clone 13 contained high levels of virus (Fig. 3a). These results show that $\sim 1 \times 10^4$ virus-specific CTL transferred in some cases up to 2 years earlier persist in an antigen-free environment and are able to provide protection against a virus infection that normal adult mice are unable to control. The data in Fig. 3a also demonstrate that long-term persistence of CTL memory without antigen is not an artefact of our Thy 1.1 to Thy 1.2 transfer system because complete protection against clone 13 challenge was also seen in the Thy 1.1 to Thy 1.1 and BALB/c to SCID groups. The long-term survival of memory CTL after transfer of purified CD8⁺ T cells into SCID mice suggests that antigen-antibody complexes do not play an essential role in maintaining CD8⁺ T-cell memory. It is possible, however, that such complexes have a role in sustaining B-cell and CD4⁺ T-cell memory^{6,9,12}.

Memory CTL persisting in the absence of antigen for >15

months were able to proliferate on re-exposure to virus and generate a virus-specific cytotoxic response. After transfer into uninfected mice, memory CTL persist at about the same numbers as the input and do not exhibit any direct *ex vivo* cytolytic activity. However, on re-exposure to virus, donor CD8⁺ T cells increased from $1-2 \times 10^6$ to $\sim 1 \times 10^7$ per spleen and acquired effector function (Fig. 3b, c). The LCMV-specific CTL response was mediated exclusively by the transferred memory CTL and the expansion was also selective for the donor CD8⁺ T-cell population. Dominant responses are a characteristic feature of memory cells and these results further demonstrate that LCMV-specific CTLp residing without antigen retain all properties associated with memory T cells^{1,5,15}.

In this study we have addressed a critical issue of immune memory, namely the role of specific antigen in maintaining immunity. A resolution of this issue is important not only for a fuller understanding of immunological memory, but also for the development of effective vaccination strategies. The main finding of our study is that the original priming antigen is not essential for the maintenance of CTL memory. It remains to be determined whether exposure to crossreactive environmental antigens, or stimulation by self-peptides involved in positive selection, plays a role in maintaining CTL memory^{1,19,20}. Even if signalling by related peptides is involved in maintaining the memory CTL, it should be noted that such cross-reactive stimuli are unable to prime virgin CD8⁺ T cells. Thus, our results show

that long-term immunity is an inherent property of memory T cells, and that, at a population level, these cells have an almost indefinite lifespan. Despite the absence of specific antigen, memory CTL continued to express higher levels of adhesion molecules and remained poised to respond faster and control a

more virulent infection. These results clearly show that, at least in the case of CD8⁺ CTL, antigen persistence is not an essential requisite for maintaining memory. These findings suggest a re-evaluation of the current dogma that long-term immunity is dependent on continuous antigenic stimulation⁶⁻¹¹. □

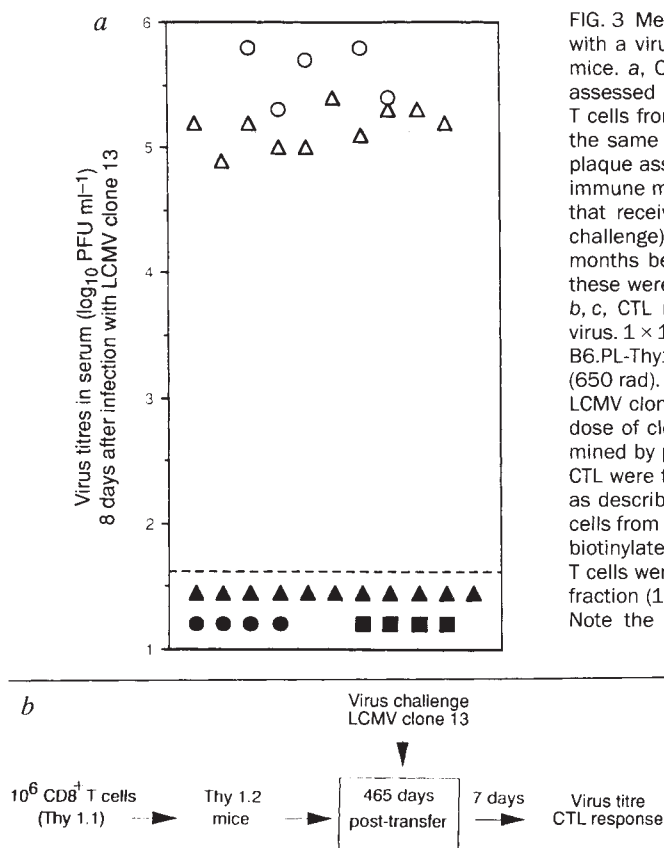
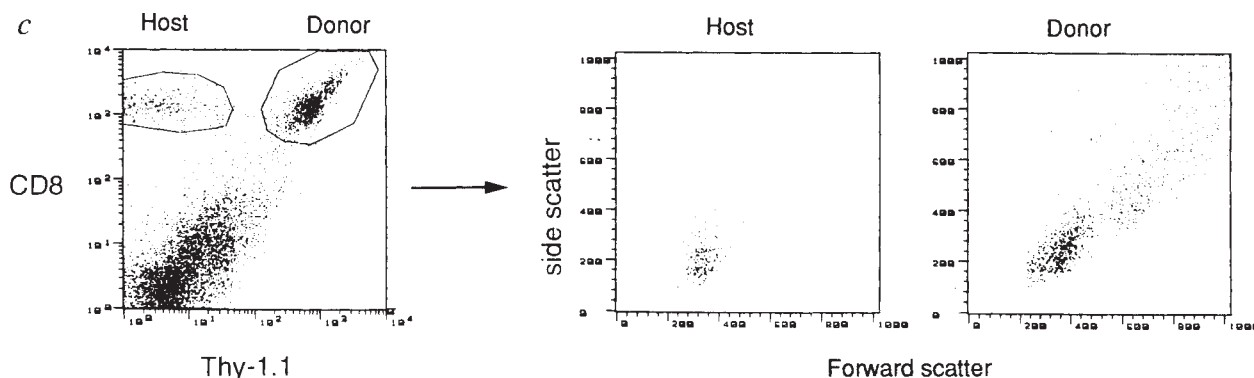


FIG. 3 Memory CTL transferred to uninfected mice confer protection against challenge with a virulent LCMV strain (clone 13) that causes persistent infection in naive adult mice. **a**, Control of virus infection. Protection against LCMV clone 13 challenge was assessed in several groups of uninfected mice that received $1-2 \times 10^6$ purified CD8⁺ T cells from LCMV-immune donors. As controls, normal adult mice were infected with the same dose (10^6 PFU) of clone 13. Virus levels in the serum were determined by plaque assay on Vero cells¹⁸. $\blacktriangle$, Thy 1.2 mice that received CD8⁺ T cells from Thy 1.1 immune mice (between 3 and 26 months before clone 13 challenge). $\blacksquare$, Thy 1.1 mice that received CD8⁺ T cells from Thy 1.1 immune mice (100 days before clone 13 challenge). $\bullet$, SCID mice that received CD8⁺ T cells from BALB/c immune mice (9 months before clone 13 challenge). $\triangle$, Normal adult Thy 1.1 or Thy 1.2 mice (note, these were fully immunocompetent mice that had not been irradiated). $\circ$, SCID mice. **b, c**, CTL response and proliferation of transferred memory cells on re-exposure to virus. 1×10^6 FACS-sorted CD8⁺ T cells (1×10^4 virus-specific CTLp) from LCMV-immune B6.PL-Thy1^a (Thy-1.1) mice were transferred i.v. into uninfected C57BL/6 (Thy-1.2) mice (650 rad). 15 months post-transfer, the recipient mice were challenged i.v. with 10^6 PFU LCMV clone 13. As controls, normal adult C57BL/6 mice were infected with the same dose of clone 13. Seven days later, virus levels in the spleen and serum were determined by plaque assay and spleen CTL activity was measured in a ⁵¹Cr-release assay. CTL were typed using anti-Thy-1.1 (ICN) or anti-Thy-1.2 (Cedarlane) plus C' (Cedarlane) as described¹⁵. To characterize further the cells responding to viral challenge, spleen cells from the clone 13-infected transfer recipients were stained with FITC-anti-Thy 1.1, biotinylated anti-CD8, and PE streptavidin, and total numbers of host and donor CD8⁺ T cells were determined (**c**). Before virus challenge, donor cells comprised only a small fraction (1–2%) of the total spleen cells ($\sim 1-2 \times 10^6$ Thy 1.1 CD8⁺ T cells per spleen). Note the substantial expansion of Thy 1.1⁺ CD8⁺ T cells on re-exposure to virus (7 days post-infection). The sample shown in **c** contained 1.2×10^7 Thy 1.1⁺ CD8⁺ cells. The size of the donor and host CD8⁺ T cells was also analysed on a FACScan using forward versus side scatter parameters. Note the selective activation (increased size and granularity) of the memory CD8⁺ T cells.

Group	Mouse number	Treatment	CTL response Specific killing on MC-57 (H-2 ^b) targets (%)					Virus titre PFU per ml or organ	
			Infected			Uninfected		Serum	Spleen
			50:1	17:1	5:1	50:1	17:1		
Thy 1.1 → 1.2 CD8	1	C'	47	24	9	1	0	<50	<50
		α Thy 1.1 + C'	3	1	1	2	1		
		α Thy 1.2 + C'	44	18	8	1	0		
	2	C'	63	38	18	1	0	<50	<50
		α Thy 1.1 + C'	2	3	1	1	0		
Normal 1.2	1	α Thy 1.2 + C'	60	37	14	2	0	3.5×10^5	6.5×10^6
		C'	4	3	3	2	0		
	2	C'	7	2	0	0	0		



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Virus-specific CD8⁺ T-cell memory determined by clonal burst size

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ALTHOUGH some viruses, particularly the herpes viruses, may never be eliminated from the body¹, others like influenza A, regularly reinfect humans and boost waning crossreactive CD8⁺ T-cell immunity². Prolonged T-cell memory is found for viruses that are unlikely to be re-encountered and which do not persist in the host genome^{3–5}, indicating that CD8⁺ T-cell memory might be independent of continued (or sporadic) antigenic exposure. A feature of virus-specific CD8⁺ T-cell memory is that antigen-specific cytotoxic T-lymphocyte precursors (CTLp) are greatly increased and remain high throughout life. The idea that persistence of the inducing antigen is essential is based on experiments in which adoptively transferred CD8⁺ memory T cells could not be detected for more than a few weeks in naive recipient mice without secondary challenge^{6,7}. Here we show that restimulation of such chimaeric mice with an inducing Sendai virus antigen increases the clonal burst size more than 7-fold within 8 days, making memory CTLp easier to detect in the longer term. We find that Sendai-virus-specific CTLp are maintained for >250 days in irradiated uninfected recipients, including reconstituted β_2 -microglobulin^{-/-} mice. To determine whether a source of viral peptide can persist after primary infection, we gave Sendai-virus-specific Thy1.1⁺ memory spleen cells to naive mice that had been minimally depleted of Thy1.2⁺ T cells, or to comparable recipients that had recovered from infection with Sendai virus or influenza virus. Although antibody against Sendai virus was never found in the naive recipients, Sendai-virus-specific CD8⁺ memory T cells were maintained equally well in each case for >100 days after cell transfer. We find no evidence for persisting depots of viral protein that might

TABLE 1 Antigen-induced proliferation of CTLp in irradiated recipients

Experiment	Recipient β_2 m Status	Secondary challenge	Reciprocal of CTLp Frequency*
1	+/+	SNP*	6,200
	+/+	FNP*	900,000
2	+/+	SNP	1,800
		FNP	13,000
	-/-	SNP	12,000
		FNP	12,600

Experiment 1: Recipient B6 Thy1.2 mice were lethally irradiated (950 rads) and injected next day with T-cell-depleted bone marrow from Thy1.2⁺ mice plus 2.0×10^7 immune spleen cells (30% CD8⁺) from congenic Thy1.1⁺ mice primed with Sendai virus 3 months previously. The frequency of Sendai virus-specific CTLp was 1/1,060, so each recipient was given ~20,000 CTLp. Spleen populations had been depleted of most of the B cells. Experiment 2: ATx, lethally irradiated (950 rad) B6^{+/+} and β_2 m^{-/-} recipient-mice were reconstituted with Thy1.2-depleted B6 or β_2 m^{-/-} bone marrow respectively, and given 1.5×10^7 Sendai-virus-primed (1 month previously) immune spleen cells from (B6 $\times$ 129) F1 donor mice. Spleen cells were first subjected to 3 cycles of depletion with Dynabeads (Dyna, Oslo, Norway): (1) TIB120 anti-class II MHC glycoprotein, followed by anti-rat Ig Dynabeads; (2) anti-mouse Ig Dynabeads; (3) anti-CD4 (H129), followed by anti-rat Ig Dynabeads. Donor cells were phenotyped using FACS as 62% CD8⁺, 0% CD4⁺ and 8% B220⁺. The 30% of cells that were not identified were the same size (forward light scatter) as the CD8⁺ set. About 5% of them were more granular (90° scatter), a morphology characteristic of natural killer cells or $\gamma\delta$ T cells. Some were CD4⁺ T cells with either blocked or downregulated CD4, as we consistently found CD4⁺ lymphocytes (1–3%) in the spleens of these ATx irradiated recipients. The frequency of Sendai-virus-specific CTLp was 1/250, so each recipient was given ~6 $\times$ 10⁴ virus-specific CTLp.

* Mice were injected with 10⁷ PFU of recombinant vaccinia virus expressing the nucleoprotein of Sendai virus (SNP) or influenza-A virus (FNP), which provide the epitopes recognized by CD8⁺ CTL-specific for both viruses in H-2^b mice^{13,14}. The SNP construct was prepared according to ref. 15. Viruses were injected 14 days after reconstitution with bone marrow, and spleens assayed for CTLp frequency after a further 8 days.

feed into the endogenous processing pathway and maintain virus-specific CD8⁺ T-cell memory.

The mouse pathogen parainfluenza type-1 Sendai virus was used to test CD8⁺ T-cell memory. Peripheral lymphoid tissue from naive controls generally contains <1:250,000–1:10⁶ CTLp specific for Sendai virus, and primary virus-specific CTL activity cannot be demonstrated after restimulation in bulk culture⁸. After a single, intranasal (i.n.) infection, memory CD8⁺ CTLp are detectable in spleens of C57BL/6J mice for life. Typical CTLp frequencies (expressed as the reciprocal) are: 15 months, 3,000–11,000; 22 months, 4,700, which is similar to values found within 3 months of primary stimulation⁸. Analysis by polymerase chain reaction (unpublished data) failed to demonstrate any persistence of the viral genome in the lungs or spleens of such mice for more than 3 weeks after i.n. infection. After 4 weeks the animals were isolated in a BL3 facility; naive animals isolated in open cages did not sero-convert, although exposure to a single Sendai virus particle causes infection and development of an antibody response. The primed mice are therefore not being rechallenged from an exogenous source. Any persisting antigen maintaining CD8⁺ T-cell memory must be in protein depots, perhaps as antigen-antibody complexes on the surface of follicular-dendritic cells (FDC)^{9,10}, although it is not clear how such protein could be reprocessed to provide peptide through the endogenous pathway. There was no CTL response or T-cell proliferation when memory spleen cells from Sendai-virus-primed mice were cultured without virus or irradiated virus-infected stimulator cells (data not shown).

To establish whether inducing antigen needs to be present to maintain CD8⁺ T-cell memory^{6,7}, we enriched spleen cells from Sendai-virus-primed donors for CD8⁺ CTLp and transferred

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them into lethally irradiated mice which were given T-cell-depleted bone marrow simultaneously. The recipients were normal Thy1-different or adult thymectomized (ATx) class I MHC β_2 -microglobulin-disrupted ($\beta_2m^{-/-}$; refs 11, 12) and control $\beta_2m^{+/+}$ mice. Some from each group were challenged 14 days later with a recombinant vaccinia virus expressing the antigenic Sendai virus nucleoprotein (SNP); an influenza construct¹³⁻¹⁵ (FNP) was used as control. There is no crossreactivity in the CD8 $^{+}$ CTL response to these two viruses. Stimulation with SNP, but not FNP, greatly increased CTLp frequency in recipient spleen within 8 d in normal and ATx $\beta_2m^{+/+}$ recipients (Table 1). However, Sendai-virus-specific CTLp frequencies were identical for the ATx $\beta_2m^{+/+}$ mice given FNP, and for the $\beta_2m^{-/-}$ recipients injected with FNP or SNP (experiment 2, Table 1). Thus, there was no establishment of class I MHC $^{+}$ antigen-presenting stimulator cells of donor origin in the $\beta_2m^{-/-}$ mice. Evidently further antigen challenge increases the clonal burst size ($>7\times$) for Sendai-virus-specific CD8 $^{+}$ CTLp, but only if appropriate antigen-presenting cells are provided by the recipient or by donor bone marrow.

The acute clonal expansion seen for Thy1.2 $^{+}$ recipients challenged with SNP (experiment 1, Table 1) is reflected by the rise in Sendai-virus-specific Thy1.1 $^{+}$ CTLp for more than 8 months after secondary challenge (Table 2). But Thy1.1 $^{+}$ donor T cells were also found during this interval in FNP controls. Although these chimaeric animals would have reconstituted their own Thy1.2 $^{+}$ T-cell response 3–4 weeks after bone marrow transfer¹⁶, there was no evidence of Sendai-virus-specific Thy1.2 $^{+}$ CTLp, and mice given FNP did not seroconvert to Sendai virus as a consequence of exposure to endogenous antigen. Similarly, Sendai-virus-specific CTLp that were not restimulated maintained equally well in ATx $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ recipients (Table 3). The increase in spleen CTLp on days 14–50 is typical for

such 'parking' experiments, and probably reflects proliferation (~ 56 -fold) that is independent of exposure to exogenous antigen¹⁷.

Although we passaged spleen populations enriched for the CD8 $^{+}$ subset, recipients may have acquired a few FDC (or macrophages) carrying viral protein. This is an unlikely explanation for the results in Tables 2 and 3 as restimulation of memory CTLp after adoptive transfer depends on antigen dose⁷. Also, the claim that there is no virus-specific memory without the continued presence of viral peptide is based on experiments using unseparated immune spleen cells and lymphocytic choriomeningitis virus, which infects T cells and causes persistence^{7,18}.

Conversely, is memory enhanced by transfer of CTLp into mice primed with Sendai virus? We gave donor Thy1.1 $^{+}$ Sendai-virus-specific memory CTLp mice that had been partially depleted of Thy1.2 $^{+}$ T cells¹⁹ after recovery from infection with Sendai virus or influenza virus, or that had not been infected (Table 4). CTLp maintained equally well in all three sets of recipients for >100 days. Furthermore, although we transferred whole immune spleen, no Sendai-virus-specific serum antibody was found in naive recipients. Even without T-cell enrichment, not enough viral protein or antigen-bearing FDC was transferred to maintain humoral immunity.

The evidence^{20,21} suggests that the continued presence of inducing antigen is not mandatory for long-term maintenance of CD8 $^{+}$ T-cell memory, but low-affinity crossreaction with (for example) self peptides presented by class I MHC $^{+}$ stimulators²² cannot be excluded. Although large numbers of class I MHC $^{+}$ stimulator cells had not established in $\beta_2m^{-/-}$ mice by day 14 after cell transfer (experiment 2, Table 1), we did not verify this in the long-term as we considered that this compartment would be repopulated by stem cells from $\beta_2m^{-/-}$ donor bone marrow²³. Also, the class I MHC glycoprotein on donor T cells would be present throughout, and there may be minimal class-I expression (particularly of H-2D b) on $\beta_2m^{-/-}$ cells²⁴. The Sendai-virus-specific CTL response is restricted by H-2K b , and T lymphocytes

TABLE 2 Consequences of secondary stimulation for maintenance of adoptively transferred Sendai-specific Thy1.1 $^{+}$ CTLp

Day	Infection with vaccinia construct*	Virus	Thy1.1 $^{+}$ cells in spleen	Bulk culture (day 6)		
				^{51}Cr release (10:1)	V	N
40	SNP		Per cent	%Thy1.1 $^{+}$		
			CTLp*			
96	SNP		6.3	88	49	0
			13,860	53	47	3
140	SNP		2.0	60	44	2
			6,270	59	37	4
168	SNP		2.4	53	57	4
			3,190	28	28	4
168	FNP		1.8	49	51	3
			126	100†	42	3
251	SNP		1.4	0	0	0
			257	9	33	1
251	FNP		2.0	100†	31	8
			2,965	0	1	6
251	FNP		2.0	44	51	3
			2,965	100†	57	5
251	FNP		2.0	0	1	6
			2,965	18	19	6
251	FNP		2.0	100†	23	5
			2,965	0	4	3

These are long-term results for the mice described in experiment 1 in Table 1. The mice were injected with virus 14 days after transfer of immune spleen cells, with the day shown being for the time elapsed since the secondary stimulation. Antibody titres for serum taken at day 168 (measured by ELISA using whole detergent-disrupted Sendai virus coated on plates) were IgG2a:SNP, 1:2,000, FNP 0; IgG2b:SNP 1:800, FNP 0.

* The reciprocal of the CTLp frequency for the Thy1.1 $^{+}$ cells in spleen was calculated from limiting dilution analysis for whole spleen (data not shown) and the per cent Thy1.1 $^{+}$ cells in spleen.

† Thy1.1 $^{+}$ and Thy1.1 $^{-}$ sets were sorted (using flow cytometry) from lymphocyte populations at 6 days restimulation with Sendai-virus-infected, irradiated spleen cells, and assayed (10:1) on virus-infected (V) and normal (N) MC57G (H-2 b) target cells.

TABLE 3 Survival of Sendai-virus-specific CTLp in $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ ATx recipients

Days after cell transfer	Recipient β_2m status	CD8 $^{+}$ in spleen (%)	Normal targets	Reciprocal of CTLp frequency		Bulk culture (d6)	
				Virus-infected		Specific ^{51}Cr release (%)	
				Total	CD8 $^{+}$	V	N
14	+/+	4.6	$<10^6$	200,000	9,200	27	0
	-/-	2.0	$<10^6$	200,000	4,000	34	0
50	+/+	1.6	$<10^6$	20,000	320	67	0
	-/-	1.0	10^6	20,000	200	67	0
91	+/+	3.0	350,000	19,000	570	40	8
	-/-	0.9	10^6	36,000	324	44	2
165*	+/+	0.9	$<10^6$	32,300	970	42	0
	-/-	1.2	10^6	34,900	455	50	0

Mice are described in experiment 2, Table 1. Results are shown for animals that were not restimulated with virus. Values are from spleens pooled from at least 3 mice. CD8 $^{+}$ CTLp frequencies were calculated for the per cent CD8 $^{+}$ cells and the frequencies for whole spleen (total). Serum samples from mice assayed on day 50 contained no virus-specific immunoglobulin. Most long-term antibody production in this model occurs in the bone marrow and serum antibody is never found in the absence of virus-specific plasma cells in this site (L.H., M. Sangster, R. Sealy and C. Coleclough, manuscript submitted). Using the single-cell ELISPOT assay²⁵, no plasma cells producing virus-specific immunoglobulin were found at day 50. CD4 $^{+}$ T cells in spleen were 1.8% for (+/+) and 2.2% for (-/-) recipients.

* Pooled samples from 4 (+/+) recipients, and from the one remaining (-/-) recipient were assayed at day 275. The reciprocal CTLp frequencies were: spleen, (+/+) 12,500; (-/-), 46,700; mesenteric lymph node (+/+) 574; (-/-) 870. The spleen in the surviving (-/-) mouse had >2 -fold more cells than the average count for the (+/+) animals. None of these mice had Sendai-virus-specific serum antibody.

TABLE 4 Adoptive transfer for Sendai-virus-specific Thy1.1⁺ T cells into convalescent Thy1.2⁺ mice

Days after transfer	Infection of recipient	Reciprocal of virus-specific CTLp frequency	⁵¹ Cr release for d6 bulk culture (2.5:1)	
			V	N
15	Nil	45,200	79	5
	Influenza	40,300	79	11
	Sendai	85,900	47	4
52	Nil	55,300	29	1
	Influenza	13,900	47	5
	Sendai	14,500	46	0
107	Nil	38,800	51	4
	Influenza	40,300	33	2
	Sendai	32,300	49	2

The donor Thy1.1⁺ mice had been primed one month previously by i.n. infection with Sendai virus. The ATx Thy1.2 B6 recipient mice were infected i.n. with either Sendai virus or the HK×31 influenza A virus, allowed to recover for 3 months (Sendai) or 1 month (influenza), then partially immunosuppressed by repeated treatments¹⁹ with the 30.H12 mAb to Thy1.2. Spleens were removed 15, 52 or 107 days later and stimulated *in vitro* under bulk culture or LDA conditions for 6 days. After culture, cells were treated with antibody against Thy1.2+ complement to eliminate any residual host T cells, and assayed on Sendai-virus-infected (V) or normal (N) MC57G target cells. The LDA results are for the V targets.

* Titres of Sendai-virus-specific IgG2a and IgG2b measured from the 3 sets of recipients on day 107 were: Nil, 0, 0; influenza, 0, 0; Sendai 1:750, 1:400.

act as veto cells rather than stimulators²⁵. Comparison of CTLp frequencies for $\beta_2m^{+/+}$ and $\beta_2m^{-/-}$ recipients thus indicates (Table 3) that any crossreactive stimulation by self (or other) peptides presented by class I MHC glycoproteins must be minimally dose-dependent. Recirculating memory T cells express an activated phenotype^{4,8,26,27} and are a physiologically dynamic population; whether this is a result of continuous or sporadic restimulation through the clonotypic T-cell antigen receptor is unclear.

Our results indicate that the continued presence of viral peptide is not mandatory in mice that have recovered from an acute virus infection. It remains an open question whether vaccines developed to promote antigen persistence will increase CD8⁺ CTLp and diminish response time⁴. □

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Immunoglobulin synthesis and generalized autoimmunity in mice congenitally deficient in $\alpha\beta$ (+) T cells

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THROUGH cognate B-cell–T-cell interactions and provision of cytokines, CD4⁺ T-cell antigen receptor (TCR) $\alpha\beta$ ⁺ T cells regulate immunoglobulin isotype synthesis¹. Murine IgG1 and IgE secretion is therefore substantially T-cell-dependent, whereas IgM and IgG3 secretion is not^{2,3}. Here we report that in the absence of $\alpha\beta$ T cells, B cells expand, differentiate and secrete copious amounts of antibodies of 'T-dependent' isotypes. Moreover, the antibodies are reactive towards self-antigens, as in patients with systemic lupus erythematosus, so autoantibodies of 'T-dependent' type can develop without the help of CD4⁺ $\alpha\beta$ T cells. This phenotype is not evident in mice or humans that are congenitally deficient in specific $\alpha\beta$ T-cell functions, but bears comparison with B-cell hyperactivity and autoimmunity in transplant rejection and in immunodeficiencies such as AIDS^{4,5}.

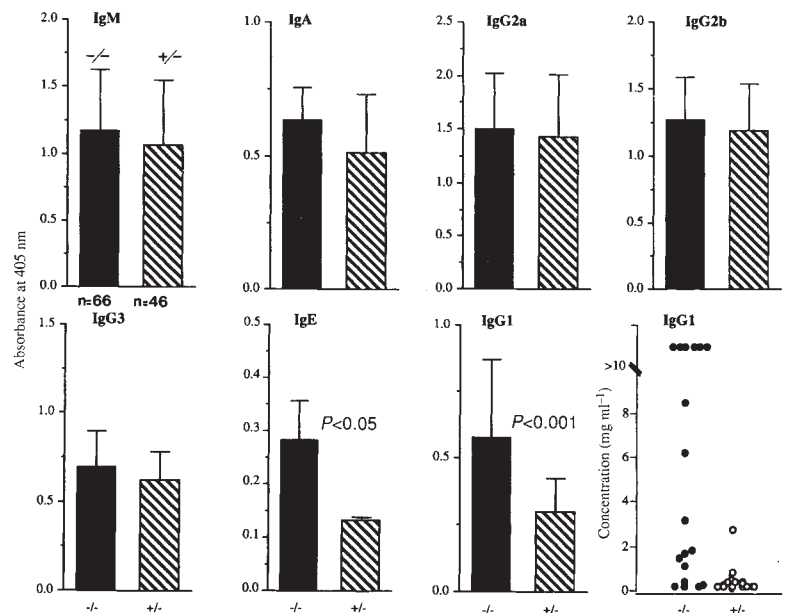
Spleens of 4–6-week-old TCR $\alpha^{-/-}$ mice maintained in pathogen-free isolators are roughly normal in size but contain high numbers of surface-IgM⁺, IgD⁺ B cells⁶. In TCR $\alpha^{-/-}$ mice housed conventionally, the spleens expand excessively relative to those of TCR $\alpha^{+/+}$ littermates (J.L.V., S.J.R., W.L., C.M., A.C.H. and M.J.O., manuscript submitted). This finding prompted us to investigate B-cell behaviour in TCR $\alpha^{-/-}$ mice.

Splenic B-cell contents of TCR $\alpha^{-/-}$ mice were determined by fluorescence-activated cell sorter (FACS) analysis of surface phenotype, using antibody B220. TCR $\alpha^{+/+}$ heterozygotes were used as controls because they are the closest genetic match to TCR $\alpha^{-/-}$ mice, and because they do not differ significantly from TCR $\alpha^{+/+}$ controls in assays of T-cell development and function (refs 6–8, and our unpublished results). The average splenic B-cell content for 52 TCR $\alpha^{-/-}$ mice was 81.5×10^6 cells (s.d. = 20.7×10^6) compared with 39.5×10^6 (s.d. = 12.9×10^6) for 44 TCR $\alpha^{+/+}$ mice. B-cell expansion in TCR $\alpha^{-/-}$ could not simply be attributed either to age (Table 1a) or to disproportionate expansion of CD5⁺ B cells, as the peritonea (Table 1b) and spleens (data not shown) of age-matched TCR $\alpha^{-/-}$ and TCR $\alpha^{+/+}$ mice contained comparable percentages of CD5⁺ B cells.

To test whether B-cell expansion in TCR $\alpha^{-/-}$ mice was accompanied by B-cell differentiation, serum immunoglobulin levels were assayed. Surprisingly, the ELISA optical density (A_{405}) readings for all immunoglobulin isotypes were at least as high for TCR $\alpha^{-/-}$ sera ($n=66$) as for TCR $\alpha^{+/+}$ sera ($n=46$) (Fig. 1). Moreover, TCR $\alpha^{-/-}$ sera commonly contained significantly higher concentrations of IgE and particularly IgG1 (Fig. 1). Again, there was no strict age correlation: 5–18-week-old TCR $\alpha^{-/-}$ mice housed together had either normal (average 0.4 mg ml^{-1}) or high ($>1 \text{ mg ml}^{-1}$) concentrations of IgG1

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FIG. 1 Immunoglobulin isotype concentrations in sera (1:100 dilution) of $TCR\alpha^{-/-}$ (solid bars) and $TCR\alpha^{+/-}$ (hatched bars) mice. The y-axis shows optical density at 405 nm which is proportional to protein concentration; mean value and s.d. for each group is indicated. Statistical significance determined using Mann-Whitney rank correlation is indicated by P value. Bottom right: individual serum IgG1 concentrations in $mg\ ml^{-1}$. METHODS. The mouse Ig isotyping ELISA Kit (Pharmingen) was used. Use of standard Igs allowed $mg\ ml^{-1}$ IgG1 concentrations to be determined for those sera that fell in the linear range of a semi-log calibration curve. Several sera were listed as $>10\ mg\ ml^{-1}$, because even after dilution they fell outside linear range and could not be quantified accurately. IgE was detected by the same assay with the reagents supplied separately.



(Table 1a). The immunoglobulin isotype analysis contrasts strikingly with that of athymic nude mice, in which serum IgG1 levels were only 4% of normal at 9 weeks of age and essentially undetectable thereafter, but could be fully rescued by thymic engraftment⁹.

The maturation of B cells in the absence of $\alpha\beta^+$ T cells raises the issue of how the B-cell repertoire is tolerized¹⁰. Three established assays (anti-DNA reactivity; anti-nuclear antibody (ANA) fluorescence; and immunoblotting of mammalian cell lysates) were used to assess autoreactivity. More than 60 $TCR\alpha^{-/-}$ mice were compared with 60 $TCR\alpha^{+/-}$ controls; 20 BALB/c and 129 mice (parental strains for $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ mice⁶); and several MRL/*lpr* mice (which spontaneously develop systemic lupus erythematosus (SLE)-like autoimmunity¹¹). Like many strains, 129 mice in particular have natural autoantibodies, enhanced by polyclonal B-cell activa-

tion, housing conditions, or age¹²⁻¹⁴. But these autoantibodies are predominantly IgM, and their occurrence is variable^{14,15}. By contrast, by 24–38 weeks of age, more than 50% of MRL/*lpr* mice have autoreactive IgG^{14,16}. Therefore, emphasis was placed on assessing the frequency of IgG autoantibodies in $TCR\alpha^{-/-}$ mice.

At a 1:100 dilution, 47% of $TCR\alpha^{-/-}$ sera ($n=68$) scored an ELISA A_{405} reading >0.4 for IgG anti-double-stranded (ds)-DNA reactivity. Although lower than many MRL/*lpr* titres, comparable reactivities were shown by only 1 in 60 $TCR\alpha$ heterozygotes, and none of the BALB/c mice (Fig. 2A). Furthermore, the difference between $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ reactivities was more apparent towards dsDNA than towards single-stranded (ss) DNA ($P=0.01$) (data not shown), emphasizing that the autoreactivity is distinct from polyclonally activated IgM reactivity that is more commonly anti-ssDNA¹⁷.

TABLE 1 B-cell expansion, IgG1 quantification and autoreactivity of $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ mice

(a) B-cell and IgG1 quantification in $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ mice						(b) Percentage of peritoneal CD5 ⁺ B cells		
Homozygous			Heterozygous			Homozygous		Heterozygous
Age (wk)	Splenic B cells ($\times 10^6$)	IgG1 ($mg\ ml^{-1}$)	Age (wk)	Splenic B cells ($\times 10^6$)	IgG1 ($mg\ ml^{-1}$)	1.13	3.98	2.93
5	92.51	0.23	6	34.17	0.39	0.78	0.55	3.33
5	96.9	ND	6	27.96	0.55	0.56	0.56	2.13
5	96.6	1.1	6	32.86	0.42			3.2
10	90.73	0.18	10	49.53	0.37			
10	91.16	1.67	10	53.37	0.17			
10	89.43	3.2	10	59.7	0.52			
			10	31.8	0.93			
15	79.25	6.34	15	43.2	ND			
15	90.24	1.5	15	35.5	ND			
			15	47.9	ND			
			15	43.4	ND			
18	85.42	>10						
18	77.27	0.44	24	49.9	0.29			
18	89.33	>10	24	42.5	ND			
18	92.06	>10	24	39.2	ND			
18	82.1	8.44	24	47	3.04			
18	85.26	0.28	24	43.1	ND			
18	87.64	0.23						
26	86.01	>10	40	63.4	2.77			
26	72.22	1.83	40	49.5	0.77			
32	84.66	>10						
32	81.94	>10						

(c) Response of $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ mice in assays for B-cell expansion and autoreactivity			
Assays	$TCR\alpha^{-/-}$ mice (%)	$TCR\alpha^{+/-}$ mice (%)	
IgG1 $> 2.5\ mg\ ml^{-1}$	57 ($n=49$)	4 ($n=46$)	
Anti-dsDNA	47 ($n=68$)	1.7 ($n=60$)	
Immunoblot	50 ($n=34$)	11 ($n=18$)	
ANA	81 ($n=16$)	29 ($n=14$)	
	$TCR\alpha^{-/-}$ mice	$TCR\alpha^{+/-}$	
Positives	($n=16$)	(n=14)	
4/4 assays	8	0	
3/4 assays	4	1	
2/4 assays	1	0	
1/4 assays	1	3	
0/4 assays	2	10	

ND, not determined.

More than 80% of $\text{TCR}\alpha^{-/-}$ sera ($n=16$) scored positive for IgG ANA by immunofluorescence. Different sera showed qualitatively different patterns (Fig. 2B), some similar to MRL/*lpr*, although again reactivity was usually lower. Reactivity was shown by 28% of $\text{TCR}\alpha^{+/-}$ sera ($n=14$) but was relatively weak

(Fig. 2B). Likewise, serum from B10.BR mice (a parent strain to MRL/*lpr*) was frequently weakly reactive (Fig. 2B).

Immunoblot reactivity towards mammalian cell proteins was shown by 50% of the $\text{TCR}\alpha^{-/-}$ sera, compared with 11% of $\text{TCR}\alpha^{+/-}$, 0% of BALB/c and 20% of 129 (Fig. 2C, a). As reactivities of several $\text{TCR}\alpha^{-/-}$ sera resembled those of MRL/*lpr*, primarily directed against small nuclear ribonucleoproteins (snRNPs), four $\text{TCR}\alpha^{-/-}$ sera were assayed directly for their capacity to immunoblot murine snRNPs¹⁸. Reactivity to snRNP protein A and a weak reactivity to snRNP protein B were appar-

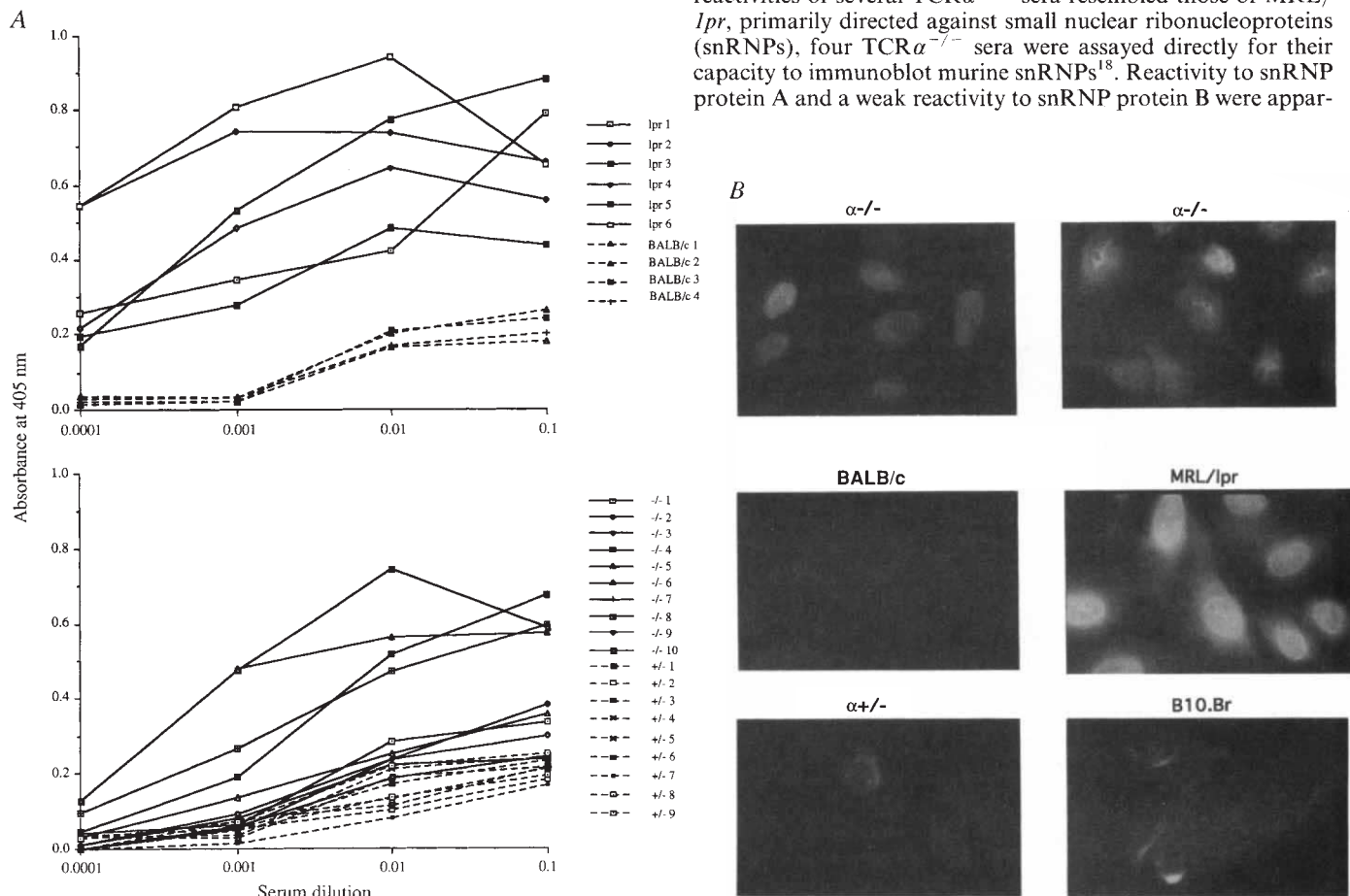


FIG. 2 Autoreactivity of sera from $\text{TCR}\alpha^{-/-}$ and control mice. A, Examples of serum IgG reactivity towards dsDNA as measured by ELISA. Genotypes (MRL/*lpr*, BALB/c, $\text{TCR}\alpha^{-/-}$, and $\text{TCR}\alpha^{+/-}$) are indicated. Top: solid lines, MRL/*lpr*; dashed lines, BALB/c; bottom: solid lines, $\text{TCR}\alpha^{-/-}$; dashed lines, $\text{TCR}\alpha^{+/-}$. B, Examples of immunofluorescent antinuclear antibody reactivities of $\text{TCR}\alpha^{-/-}$, MRL/*lpr*, BALB/c, B10.Br and $\text{TCR}\alpha^{+/-}$ sera. C, Immunoblots demonstrating reactivity of sera toward human cell proteins (a), and purified murine snRNPs (b). In a, sera (1:100) from 7 unprimed $\text{TCR}\alpha^{-/-}$ and 4 unprimed $\text{TCR}\alpha^{+/-}$ mice were reacted against membrane-immobilized HeLa lysates. b (from left), Sera from 4 HeLa-reactive $\text{TCR}\alpha^{-/-}$ mice, an MRL/*lpr* mouse and a BALB/c mouse ($\pm$ DNase I treatment) reacted with snRNPs; two human sera ($\pm$ DNase I) reacted with snRNPs¹⁸. Arrows A, B and D represent proteins A, B and D of snRNPs, respectively.

METHODS. A, Microtitre plates were coated with $10 \mu\text{g ml}^{-1}$ antigen in NaHCO_3 buffer (pH 9.6) overnight at 4°C . Two independent lots of S1 nuclease-treated calf thymus DNA (type V; Sigma) were used as antigen (heat-denatured calf thymus DNA was used as ssDNA; data not shown). Wells were washed (PBS + 0.05% Tween 20) and blocked (1% BSA in PBS blocking buffer) for 1 h at 37°C , then incubated with $50 \mu\text{l}$ of serum samples diluted in blocking buffer for 2 h at 37°C . Wells were washed and incubated with alkaline phosphatase-conjugated goat anti-mouse

Ig γ -chain antibody for 2 h at 37°C , followed by incubation with *p*-nitrophenylphosphate substrate. B, Hep-2 cell substrates were incubated with $100 \mu\text{l}$ serum (1:20), washed, developed with FITC-conjugated goat anti-mouse Ig γ -chain, and assayed by fluorescence microscopy.

ent (Fig. 2C, b). The anti-snRNP reactivity (also detectable by ELISA and by immunoprecipitation (not shown)) was DNase I-resistant (Fig. 2C, b), suggesting that it is directed against protein rather than nonspecifically bound DNA. By contrast, DNase I treatment can abolish anti-snRNP reactivity in many human SLE sera (Fig. 2C, b).

In summary, after 5 weeks of age (the youngest tested) ~50% of 68 $TCR\alpha^{-/-}$ sera showed IgG autoantibodies by all three criteria (Table 1c). Although $TCR\alpha^{+/-}$ and 129 sera were occasionally positive, it was rare that they scored positive in more than one assay. Of 16 $TCR\alpha^{-/-}$ mice assayed for excess IgG1, anti-dsDNA, ANA and immunoblotting, 50% were positive for all four, and 75% for three of four (Table 1c). By contrast, 71% of 14 $TCR\alpha^{+/-}$ sera were negative for all four parameters, and 93% were negative for at least three of the four. Therefore the loss of the $TCR\alpha$ -chain gene predisposes an animal to B-cell expansion, secretion of 'T-dependent' immunoglobulin isotypes, and generalized autoimmunity. This phenotype is not shown by other models of T-cell deficiency, for example athymic nude mice or mice lacking $CD4^{+}$, $\alpha\beta^{+}$ T cells (as a result of a mutation in major histocompatibility complex class II), in which serum IgG1 levels are reduced between 10- and 100-fold, and in which there is no measurable ANA or anti-dsDNA reactivity¹⁹.

IgG1 and IgE secretion are induced in activated B cells by the interleukin IL-4, produced under normal circumstances by $CD4^{+}$, $\alpha\beta^{+}$ Th2 cells¹. The considerable amounts of IgG1 and IgE suggested that in $TCR\alpha^{-/-}$ mice there might be an alternative source of IL-4. This was also indicated by comparable expression on $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ B cells of CD23 (for which IL-4 is the most potent known inducer²⁰ (data not shown)). In seeking a potential source of IL-4, we considered $\beta\alpha^{-}$ T cells and $\gamma\delta$ T cells. Both cell types expand excessively in $TCR\alpha^{-/-}$ mice ($\gamma\delta$ T cells to an average of 11% of splenocytes; J.L.V. *et al.*, manuscript submitted), but not in nude or $CD4^{-/-}$ mice which lack the observed phenotype. Other sources, mast cells or basophils, should occur comparably in $TCR\alpha^{-/-}$, nude or $CD4^{-/-}$ mice. FACS analysis directly *ex vivo* showed that an average of 27% (range 14–40%) of $TCR\alpha^{-/-}$ $\gamma\delta$ cells expressed the activation marker CD69, suggesting that many were activated *in vivo*. In the present study, $\gamma\delta$ T cells were studied further.

To test for cytokine production, we followed the stimulation of CT4S and CTLL-2 proliferation by IL-4 and IL-2, respectively, and the inhibition of WEHI-279 by interferon (IFN)- γ (Fig. 3a). These specificities were confirmed by the use of blocking anti-cytokine antibodies (data not shown). High levels of IL-4 were detected in supernatants from $TCR\alpha^{-/-}$ splenocyte cultures stimulated with purified protein derivative (PPD), a stimulus for $\gamma\delta$ T cells, whereas IL-4, IL-2 and IFN- γ were detected in $TCR\alpha^{+/-}$ supernatants (Fig. 3a). IL-4 production was also demonstrated by FACS-sorted $\gamma\delta$ T cells (purity >99%) from $TCR\alpha^{-/-}$ mice; this was more than doubled by $\gamma\delta$ cell co-culture with FACS-sorted B cells (purity >99%) and was further provoked by pokeweed mitogen (PWM), which enhances T-dependent B-cell activation (Fig. 3b). $\gamma\delta$ T-cell IL-4 secretion was comparable to that of $\alpha\beta$ T cells purified from $TCR\alpha^{+/-}$ mice and co-cultured with $TCR\alpha^{-/-}$ B cells (Fig. 3b). (Note that the T cells used in these assays probably constitutively secreted IL-4 because of activation provoked by antibody-mediated cell sorting.) IL-4 secretion by $\gamma\delta$ T cell–B-cell co-cultures suggests that Th2 activity is not restricted to a distinct $\alpha\beta$ T-cell lineage, but rather is a functional potential of several cell types.

A capacity of $\gamma\delta$ T cells to 'help' is consistent with the reported $\gamma\delta$ T-cell help of anti-DNA autoantibody-producing B cells in patients with active lupus nephritis²¹. It may be a manifestation *in vivo* of many cases of direct $\gamma\delta$ T-cell–B-cell interaction *in vitro*²². But these interactions may not involve binding of gp39 to CD40, which seems to be essential for cognate $CD4^{+}$ $\alpha\beta$ T-cell–B-cell interactions²³ as we could not detect gp39 on $TCR\alpha^{-/-}$ $\gamma\delta$ T cells. A pathway has recently been described

in which switching of pre-activated murine B cells to IgG1 can be promoted by cytokines without cognate $\alpha\beta$ T-cell–B-cell interactions²⁴. Although a gp39-independent pathway may not be effective in conventional responses to exogenous antigens, it might explain B-cell responses in several clinical conditions.

The development in $TCR\alpha^{-/-}$ mice of 'T-dependent' isotypes with autoreactive SLE-like specificities may be consistent with the two-stage model proposed for murine models of SLE, such as MRL/*lpr* (refs 14–18, 25). In stage one, polyclonally activated B cells produce low-affinity IgM autoantibodies. In the second stage, the autoantibodies mature to high-affinity 'T-dependent' isotypes, characterized by antigen-driven, oligoclonal affinity maturation. In MRL/*lpr* mice, this is assumed to be driven by antigen-specific $CD4^{+}$ $\alpha\beta$ T cells^{18,25}, consistent with abrogation

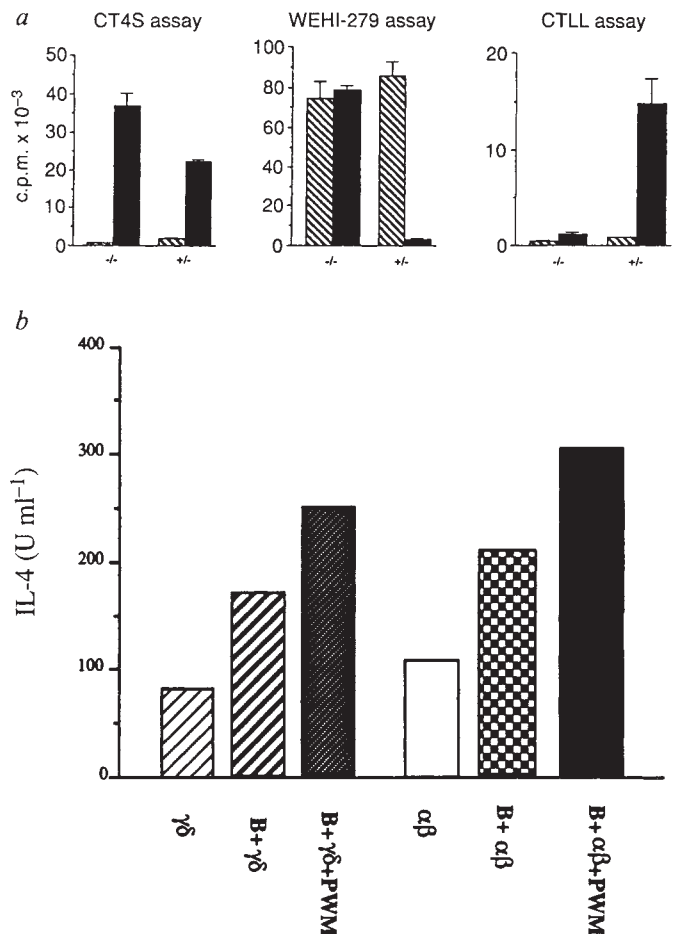


FIG. 3 a, Cytokines in supernatants of splenocytes from $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ mice, measured by the capacity to alter proliferation of 'read-out' cell lines; proliferation is quantified by 3H -thymidine incorporation. b, IL-4 production by highly purified FACS-sorted $\gamma\delta$ T cells from $TCR\alpha^{-/-}$ mice.

METHODS. a, Supernatants (diluted 1:2) of splenocytes (10^5 per well) cultured in the presence (solid bars) or absence (hatched bars) of PPD for 72 h were assayed for stimulation (increased proliferation) of CT4S cells (IL-4) or CTLL-2 cells (IL-2), or inhibition (decreased proliferation) of WEHI-279 (IFN- γ). A dose-response curve using recombinant IL-2 (Glaxo), IL-4 (Gibco) and IFN- γ (Sigma) was obtained in each bioassay. b, $TCR\alpha^{-/-}$ and $TCR\alpha^{+/-}$ splenocytes were stained with PE or FITC-conjugated mAbs (H57-anti-TCR β , GL3-anti TCR $\gamma\delta$ and anti-mouse polyvalent Ig) and sorted using a FACStar. Gates were set by forward and side scatter of unstained cells. 20,000 purified $\gamma\delta$ or $\alpha\beta$ T cells were cultured alone or with 20,000 purified B cells $\pm$ PWM ($1 \mu g ml^{-1}$) as indicated. Supernatants were collected on day 6 and IL-4 was detected by ELISA and converted to units per ml. Purified B-cell culture supernatants registered a background level of IL-4 that was subtracted from each of the readings.

of autoantibody development by genetic depletion of CD4⁺ $\alpha\beta$ T cells²⁶. However, development of the phenotype in TCR $\alpha^{-/-}$ mice shows that CD4⁺ $\alpha\beta$ T cells are not essential for autoantibody maturation. Thus, $\alpha\beta$ T cells that have escaped tolerance may not be the driving force for 'T-dependent' autoantibodies in all clinical situations. Instead, deregulated cytokine provision to B cells may be responsible, including IL-4 provision by either $\gamma\delta$ T cells in TCR $\alpha^{-/-}$ mice or allogeneic $\alpha\beta$ ⁺ Th2 cells in graft-versus-host reactions⁴. Alternatively, in some individuals an excess of IL-4 (or a complementary deficit of Th1 cytokines, notably IL-2) might be congenital or environmentally induced. By contrast, the failure to develop 'T-dependent' autoantibodies in nude mice, CD4 $^{-/-}$ mice, or in humans with CD40-gp39

deficiencies suggests that in these cases cytokine deregulation is not enough to drive the second stage.

A basis for cytokine deregulation in TCR $\alpha^{-/-}$ mice and in graft-versus-host reactions may be, directly or indirectly, the absence of CD8⁺ $\alpha\beta$ T-cell function. In CD8 $^{-/-}$ mice, pathological immune infiltrates in experimentally induced allergic encephalomyelitis do not undergo the periodic remission that they undergo in CD8 $^{+/+}$ mice²⁷. By extrapolation, CD8 T-cell deficiency could also contribute to B-cell hyperplasia and generalized autoimmunity in virally induced lymphopenia, such as in the BB rat²⁸, and HIV-infected individuals⁵. Interestingly, the latter cohort, like TCR $\alpha^{-/-}$ mice, frequently have unusually high numbers of lymphoid $\gamma\delta$ T cells²⁹. □

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Severe sensory and sympathetic deficits in mice lacking neurotrophin-3

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DURING development, neurotrophins help shape the nervous system by regulating neuronal survival and differentiation. Neurotrophin-3 (refs 1–5) is the most abundant neurotrophin during early development⁶. Neurons responsive to neurotrophin-3 *in vitro* include primary sensory, sympathetic^{1–4}, motor⁷, enteric¹, locus coeruleus⁸, hippocampal and cerebellar neurons (ref. 9 for example). Here we report that mice lacking neurotrophin-3 have severe deficits in sensory and sympathetic populations. These mice lack muscle spindles and show abnormal limb positions. In contrast, motor neurons, the enteric nervous system, and the major anatomical regions of the central nervous system seem to develop normally. Comparisons with mutants deficient in other neurotrophins^{10–12} or their receptors^{13–15} indicate that some neurons require more than one neurotrophin during embryogenesis and suggest that neurotrophin-3 functions by binding receptors in addition to its primary receptor trkC (ref. 16). In particular, neurotrophin-3 is essential for survival of sympathetic and sensory neurons that later become dependent on nerve growth factor or brain-derived neurotrophic factor.

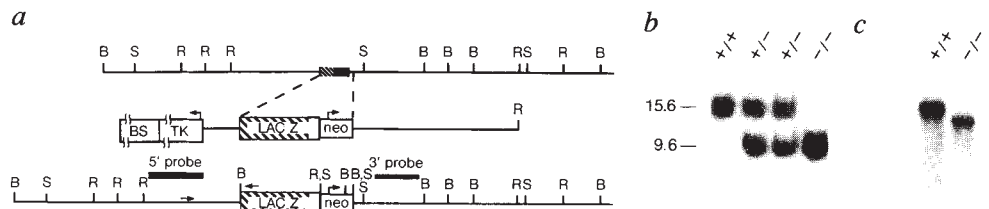
To determine the essential roles of neurotrophin-3 (NT-3) in development, we have targeted the NT-3 gene to generate mice carrying a null mutation as explained in Fig. 1. Heterozygous mice show no obvious morphological or behavioural abnormali-

ties. Matings of heterozygotes produce homozygous mutant offspring at a frequency of 16% ($n=211$), indicating that mice lacking NT-3 can develop to term. After birth, most homozygous mutants display reduced activity, abnormal limb postures and no evidence of food intake, dying within 24 h. The very small number (3 of 300 total) that survived for longer periods (7 to 15 days) showed athetotic walking movements.

NT-3 and its receptor trkC (ref. 16) are highly expressed in embryos in multiple tissues^{6,17–19}. Neuronal counts in primary sensory populations, a sympathetic ganglion and two brainstem motor nuclei are shown in Table 1. The largest losses in sensory populations are found in the cochlear ganglion, consistent with the *in vitro* responsiveness of these neurons and NT-3 expression *in vivo*²⁰; the trigeminal ganglion, consistent with NT-3 effects on ganglia from early embryos²¹; and dorsal root ganglia (DRG) (Fig. 2a, b).

The small proportion of cells in the neonatal DRGs that express trkC (ref. 22) are large neurons likely to innervate muscle spindles. The central branches of these neurons form the Ia projection to the ventral horn of the spinal cord. Mutants of *trkC* (ref. 14) show athetotic movements, a 19% cell loss of DRG neurons and a complete absence of Ia muscle afferents. In the NT-3 homozygotes, muscle spindles (Fig. 2c), whose development depends on sensory innervation²³, are absent (for example in the thigh semitendinosus muscle: wild-type: 15 ± 1 , $n=4$; mutant: 0 ± 0 , $n=3$). Moreover, staining of spinal cords shows that fibres that express p75^{LNGFR} and project to the ventral horn, likely to be the Ia projection²⁴, are missing in the mutant (Fig. 2d, e; the apparent difference in size of the two spinal cords suggests that the mutant may have additional deficits). In addition to deficits shared with the trkC mutant, NT-3 mutants are also missing additional DRG populations, because 60–80% of the normal complement of neurons are absent (Table 1). The missing cells must include neurons absent in brain-derived neurotrophic factor (BDNF)^{11,12} and/or nerve growth factor (NGF)¹⁰ mutant animals, because about 30 and 70%,

FIG. 1 Strategy and analysis for NT-3 gene targeting. **a**, The NT-3 coding exon is shown schematically with a restriction map of the flanking genomic region (top). The signal sequence (filled), pro sequences (hatched) and mature hormone (filled) are indicated. The DNA construct used for gene targeting (middle) and mutation structure (bottom) are also shown. The pBluescriptKS (BS) vector, MC1tk thymidine kinase gene (TK, see ref. 30, *Escherichia coli* lacZ gene, and PGKneo gene (neo, see ref. 30) are indicated. Restriction sites shown are *Eco*RI (R), *Bam*HI (B), and *Spe*I (S). Attached arrows indicate positions and orientations of promoters; unattached arrows designate locations of PCR primers; the probes used for DNA blot analysis are also shown. **b**, **c**, DNA blot analysis of *Eco*RI-digested tail DNA from mice having the NT-3 genotype indicated at the top of the panels. The 5' (**b**) and 3' (**c**) probes are shown in **a**.



respectively, are missing in these mutants. Staining with anti-calcitonin-gene-related peptide (CGRP) reveals that at least some axons of small, NGF-dependent cutaneous afferent neurons are present in the dorsal horn of the NT-3 mutant (Fig. 2f, g). The apparent similarity of CGRP terminal density to the wild-type is likely to represent terminal sprouting²⁵. *In vitro*, NT-3 interacts weakly with *trkA* and *trkB* (ref. 26), suggesting that the more dramatic effects seen in DRGs in NT-3-deficient compared to *trkC*-deficient mice reflect interactions of NT-3 with another neurotrophin receptor.

Sympathetic neuroblasts express *trkC* and are dependent on NT-3 (refs 27, 28). Later, sympathetic neurons lose responsiveness to NT-3, express *trkA*, and become dependent on NGF²⁷. Antibody injection or targeted disruption of the NGF gene causes a dramatic loss (70–90%) of sympathetic neurons (ref. 10 for example) and increased cell death during the perinatal period, visualized as pyknotic cells. In newborn NT-3 mutants, a considerable deficit (50%) in cell number is also found in the superior cervical ganglion (SCG) (Table 1; Fig. 3a, b), but without an increase in pyknotic neurons (not shown). Earlier loss of sympathetic precursors could reconcile these results. All surviving sympathetic neurons express tyrosine hydroxylase (TH), a marker for catecholaminergic neurons (Fig. 3c), and their fibres innervate sympathetic target organs, such as the digestive tract (Fig. 3d), although their density is reduced in some organs (not shown). Adrenal chromaffin cells are also derived from the sympathoadrenal lineage. Chromaffin cell precursors express *trkC* during development¹⁸, but seem to develop normally in NT-3 mutants (Fig. 3e, f).

Because *trkC* is the only *trk* receptor detected in enteric neural crest or ganglia^{18,19}, NT-3 is the most likely neurotrophin to support these cells¹. However, in whole mounts, the myenteric plexuses of neonatal NT-3 mutants exhibit an apparently normal network of enteric ganglia (Fig. 3g–i). Cross-sections reveal a normal location and appearance of myenteric neurons between the circular and longitudinal muscle layers (Fig. 3i). Because this system is still developing at birth, deficiencies may arise later.

Motor neuron cell counts in the brainstems of newborn mice show no obvious deficits in NT-3 mutant animals (Table 1, Fig. 2h, i), even though NT-3 is highly expressed in embryonic muscle⁷. Motor neurons express both *trkB* and *trkC* (ref. 7) and are supported *in vitro* by their ligands BDNF, NT-4/5 and NT-3 (ref. 7). *TrkB* (ref. 13), but not BDNF mutants show noticeable motor neuron deficits^{11,12}. Because NT-4/5 is not noticeably expressed in embryonic muscle, it seems unlikely to be the sole

KO1), the NT-3 coding exon was replaced, beginning at the first methionine codon, with the *E. coli* lacZ gene and the PGKneo selectable marker. Using the *Sma*I restriction site in the NT-3 coding exon² as 0.0, the genomic fragments used span (–1.85 kb to –0.35 kb) and (+0.52 kb to +10.0 kb). The D3 embryonic stem cell line was electroporated with linearized pMNT3-lacZ/KO1 and grown as described³⁰. Mutant ES clones were identified by PCR and microinjected into C57/BL6 blastocysts. The genomic structure of the mutation was confirmed using the 5' (–2.9 kb to –5.1 kb *Bgl*II) and 3' (–0.35 kb to +3.3 kb) probes.

motor neuron neurotrophin *in vivo*. Functional redundancy may explain the absence of effects on motor neurons in single neurotrophin mutants.

Some neuronal populations in the developing and postnatal brain are responsive to NT-3 *in vitro*^{8,9}. However, no obvious abnormalities were found in the cerebral cortex, hippocampus or cerebellum of the NT-3 mutant in Nissl-stained sections. Noradrenergic locus coeruleus neurons are present in the mutant and express TH (Fig. 2j), despite previously observed survival effects of NT-3 on these neurons *in vitro* and *in vivo*⁸.

In conclusion, NT-3 deficiency results in profound sensory and sympathetic deficits, including losses of neurons shown in neonates to be dependent on other neurotrophins. Consistent with this, previous results have suggested that NT-3 acts early in

TABLE 1 Neuronal counts in sensory, sympathetic and motor populations of wild-type and NT-3 mutant newborn mice

	Wild-type	NT-3 mutant	Reduction (%)
Sensory ganglia/nuclei			
Trigeminal	39,390 ± 1,693	15,274 ± 1,119	61***
Geniculate	1,548 ± 48	1,156 ± 174	25*
Vestibular	4,774 ± 554	3,644 ± 159	23
Cochlear	8,082 ± 1,132	1,284 ± 120	85***
Superior-jugular	2,427 ± 567	1,989 ± 69	18
Petrosal-nodose	7,636 ± 364	5,278 ± 482	30**
Trigeminal Mesencephalic nucleus	462 ± 71	201 ± 66	56*
C1 dorsal root	4,692 ± 626	1,761 ± 56	62*
L5 dorsal root	10,054 ± 753	2,176 ± 88	78***
Sympathetic ganglion			
Superior cervical	16,728 ± 1,366	8,775 ± 1,347	48*
Motor nuclei			
Trigeminal	852 ± 130	854 ± 66	n.s.
Facial	3,278 ± 391	3,078 ± 237	n.s.

Newborn wild-type and mutant littermates were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, embedded in paraffin, sectioned at 10 µm, and Nissl stained with 0.1% cresyl violet. Neurons having a clear nucleolus were counted in every sixth section. In all cases, three different animals were analysed per genotype. Values were not corrected and are expressed as mean number of neurons ± s.e.m. Differences were tested using a one-tailed Student's *t*-test. **P* < 0.05, ***P* < 0.02, ****P* < 0.001. C1, first cervical; L5, fifth lumbar.

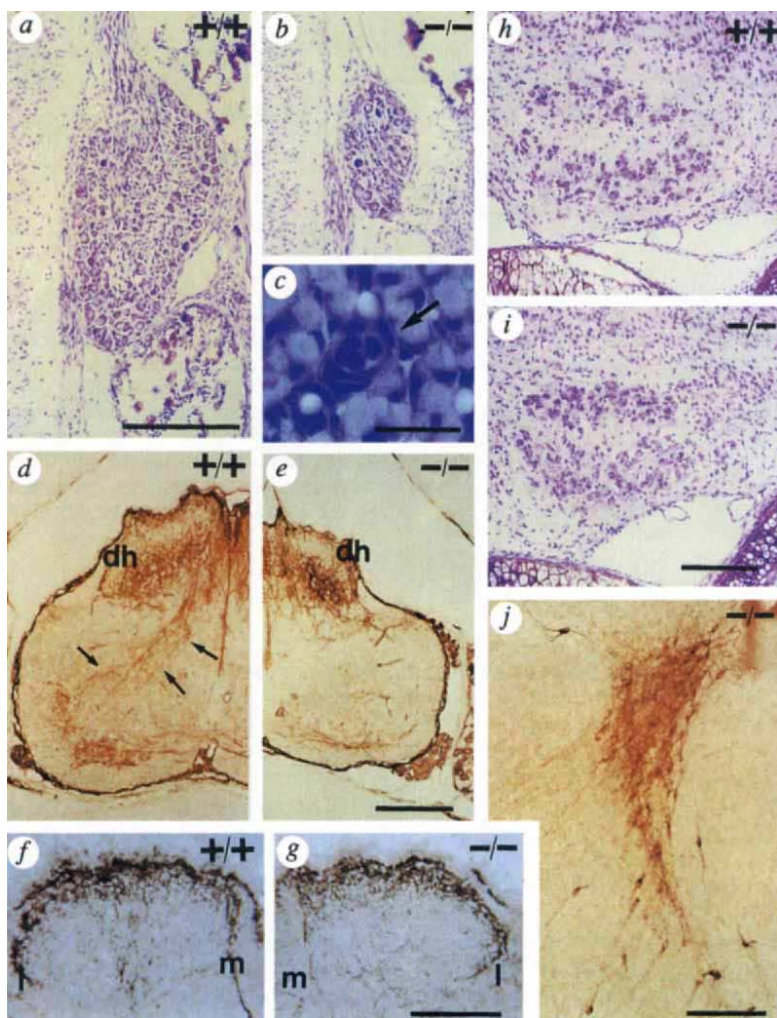
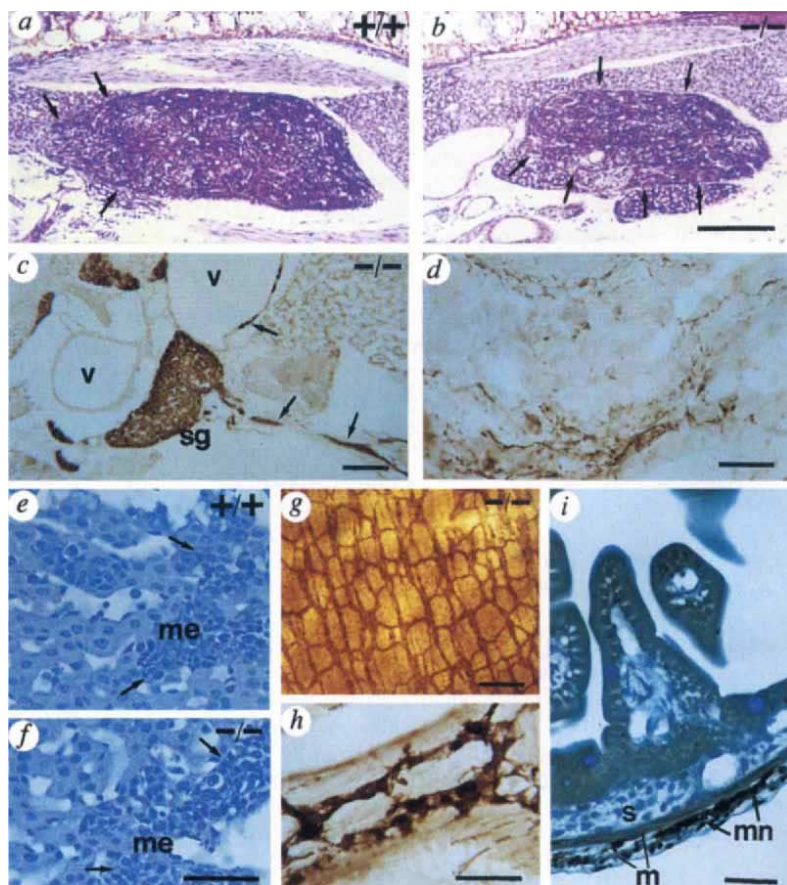


FIG. 2 Sensory and brain populations in newborn wild-type (+/+) and NT-3 mutant (-/-) mice. *a, b*, Nissl-stained sections through equivalent levels of the fifth lumbar dorsal root ganglia of wild-type (*a*) and mutant (*b*) mice, showing a considerable reduction in neuronal number in the mutant. *c*, Toluidine-blue-stained section through skeletal muscle in a wild-type animal showing a muscle spindle (arrow). Spindles, whose development is dependent on innervating sensory fibres²³, are absent in the mutant, suggesting that proprioceptive neurons are absent. *d, e*, Transverse sections through the spinal cords of wild-type (*d*) and mutant (*e*) at lumbar levels stained with antibodies to p75^{LNGFR}. Note the absence of the stained fibres projecting to the ventral horn (arrows). *dh*, Dorsal horn. *f, g*, Dorsal horn in the spinal cord of wild-type (*f*) and mutant (*g*) stained with antibodies to CGRP, indicating that at least some thermo- and nociceptors are present in sensory ganglia. *m*, Medial; *l*, lateral. *h, i*, Facial motoneucleus for wild-type (*h*) and mutant (*i*). Note the normal appearance of the mutant. *j*, Section through the locus coeruleus of a mutant mouse stained with antibodies to TH. Note presence of catecholaminergic neurons similar to those in wild-type animals (latter not shown). Scale bars, *a, b, f, g, j*: 200 μ m; *d, e, h, i*: 300 μ m; *c*: 500 μ m.

METHODS. For immunocytochemistry, fixed tissue was cryoprotected with 30% sucrose in PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4) and frozen in OCT (Tissue Tek) using dry ice. Cryostat sections (10 μ m thick) were incubated with antibodies to p75^{LNGFR}, diluted 1/3,000, or CGRP (Amersham), diluted 1/3,000, followed by detection using the avidin-biotin-peroxidase (ABC) method (Vector Labs) as described elsewhere¹¹. Brain immunocytochemistry was done as previously described¹¹.

FIG. 3 Effects on the newborn sympathetic and enteric nervous systems in wild-type (+/+) and NT-3 mutant (-/-) mice. *a, b*, Nissl-stained sections through the superior cervical ganglion of wild-type (*a*) and NT-3 (*b*) mice. In the mutant there is a considerable reduction in cell number. *c*, Ganglion of the sympathetic paravertebral chain in an NT-3 animal immunostained for TH showing that the remaining neurons in the mutant are catecholaminergic. Sympathetic ganglion (*sg*), blood vessels (*v*), and sympathetic fibres (arrows) are indicated. *d*, TH-positive sympathetic fibres are also found in sympathetic targets in mutant mice. Here, catecholaminergic fibres are shown innervating the wall of the stomach. *e, f*, Sections through the adrenal gland of wild-type (*e*) and mutant (*f*) showing the presence of medullary chromaffin cells (arrows) in both cases. *me*, Adrenal medulla. *g*, Whole-mount of the duodenum of an NT-3 mouse stained with antibodies to neuronal-specific enolase, showing the presence of a myenteric plexus, similar to that in wild-type mice (latter not shown). *h*, Myenteric plexus in a section through the stomach wall of a mutant. *i*, Section through the whole-mount shown in *g*, illustrating presence of myenteric neurons. Morphology appears normal. Submucosa (*s*), muscle layers (*m*) and myenteric neurons (*mn*) are present. Scale bars, *a, b, e, f, h*: 200 μ m; *d, i*: 100 μ m; *c, g*: 500 μ m.

METHODS. TH immunostaining using antibodies from Eugene Tech., diluted 1/3,000, was done as described¹¹. For whole-mount immunocytochemistry using antibodies to neuronal-specific enolase (Incstar Co.), small intestines were dissected, permeabilized in 1% Triton X-100 in PBS and processed as described for sections¹¹. Labelled whole mounts were embedded in glycol-methacrylate (JB4, Polysciences Inc.), sectioned at 4 μ m and counter-stained with 1% toluidine blue.



embryogenesis on transiently responsive precursors²⁷⁻²⁹. Because the phenotype of *trkC* mutants¹⁴ is less severe than that of NT-3 mutant mice, NT-3 actions are likely to be mediated through other *trk* receptors as well as through *trkC*, as suggested by *in vitro* data. □

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Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor

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FLOWER colour is determined primarily by the production of pigments, usually anthocyanins or carotenoids, but the shade and intensity of the colour are often changed by other factors such as vacuolar compounds, pH and metal ions^{1,2}. Pigmentation can also be affected by the shape of epidermal cells, especially those facing prospective pollinators^{3,4}. A conical shape is believed to increase the proportion of incident light that enters the epidermal cells,

enhancing light absorption by the floral pigments, and thus the intensity of their colour. We have identified a gene (*mixta*) that affects the intensity of pigmentation of epidermal cells in *Antirrhinum majus* petals. The cells of the corolla lobes fail to differentiate into their normal conical form in *mixta* mutants. We have cloned the *mixta* gene by transposon tagging; its sequence reveals that it encodes a Myb-related protein that probably participates in the transcriptional control of epidermal cell shape.

To examine loci that influence floral pigment intensity, we outcrossed a *mixta* line⁵ to a fully pigmented *Antirrhinum* stock (JI:522) to reselect palely pigmented individuals (that is, those carrying *mixta*) in the F₂ generation (Fig. 1*b* compared with *a*). Among these we observed some novel individuals showing somatic instability (Fig. 1*c*), with small sites and sectors of clonally related, darker cells on a background of less intensely pigmented cells (Fig. 1*d*). The progeny of these somatically unstable plants showed a high proportion (10-20%) of wild-type (darkly pigmented) plants. This genetic behaviour is characteristic of the Tam4 transposable element⁶; crossing the original *mixta* line with the wild-type stock had apparently activated a somatically stable transposon which had caused the original *mixta* mutation. One copy of Tam4 was present in DNA from *mixta* lines but

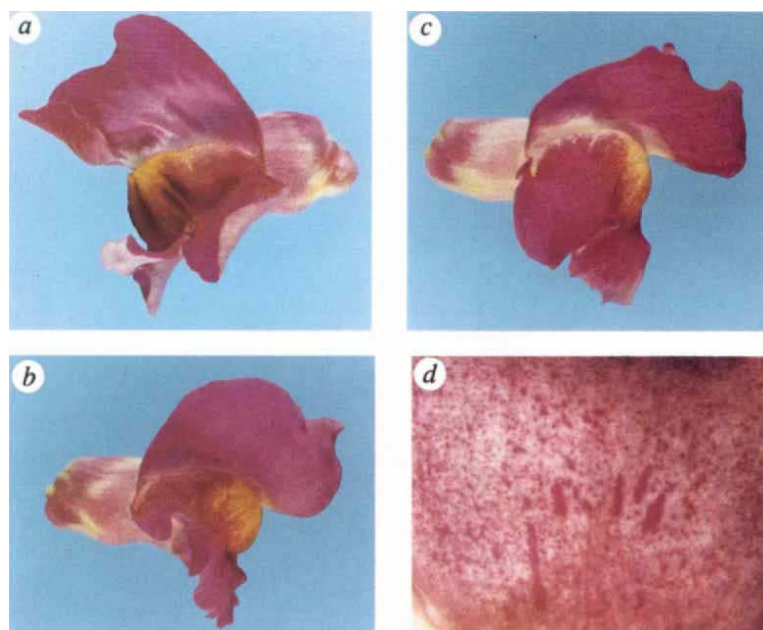
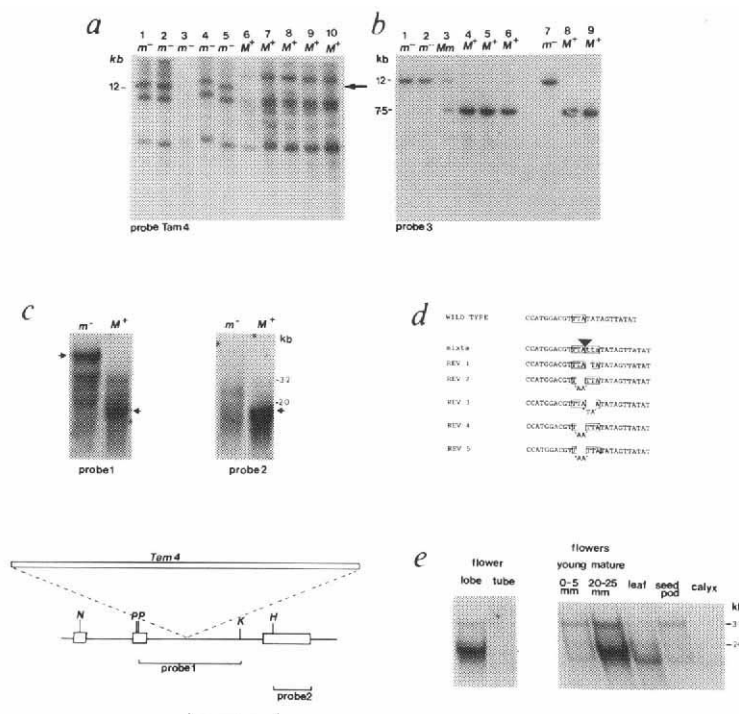


FIG. 1 Phenotype of the *mixta* mutation. *a*, Wild-type *Antirrhinum* flower showing a dark, velvet-like inner epidermis, which is particularly pronounced towards the outer edge of the petal lobes. *b*, Flower showing *mixta* phenotype with pale, dull lobes. *c*, Flower showing somatically unstable *mixta* phenotype with darker, velvet-like spots on an otherwise pale, dull inner epidermis. *d*, Magnification of lobe tissue from *c* showing the darker revertant sites on the paler background.

FIG. 2 Isolation of the *mixta* gene by transposon tagging. **a**, Southern blot of genomic DNA from *mixta* lines (m^-) and homozygous, germinal revertants (M^+) cut with *Bgl*II and probed with the *Antirrhinum* transposon Tam4. A 1.2-kb band present in the *mixta* lines and absent in the revertants is indicated by an arrow. **b**, Southern blot of genomic DNA from *mixta* lines (lanes 1, 2 and 7) and from progeny of two independent germinal revertants. The parent of one family was heterozygous (progeny shown in lanes 3, 4, 5, 6) and the parent of the other family was homozygous *Mixta* (progeny shown in lanes 8 and 9). The DNA was cut with *Bgl*II and probed with the sequences flanking Tam4 from the clone isolated from the *mixta* line (clone 208). **c**, Bottom, restriction map of the *mixta* locus showing exons of the *mixta* gene (boxes) and the position of Tam4 within the second intron in the mutant line, compiled from sequence data from the clone of the Tam4 insertion in *mixta*, the wild-type *mixta* locus and the *mixta* cDNAs. Top, Northern blots of poly(A)⁺ RNA (7 μ g) probed with probe 1 (left panel) and showing a *mixta* transcript of 1.2 kb (right arrow) in wild-type flowers (M^+) which was absent in *mixta* lines (m^-). Hybridization to other transcripts is due to sequences in the probe that encode the conserved Myb DNA-binding domain which is present in several *Antirrhinum* plant genes⁸. Instead, in *mixta* lines, a larger transcript of ~5 kb was apparent (left arrow). Right panel, probe 2 hybridized only to the 1.2-kb transcript (arrow) in wild-type (M^+) flowers but not to the larger transcript in *mixta* (m^-) flowers. Therefore Tam4 is transcribed but the transcript terminates within the element so that the 3' *mixta* sequences of exon 3, encoding part of the DNA-binding domain and the C-terminal (potential activator) domain are not transcribed. By analogy with other Myb proteins⁹, we conclude that insertion of the transposon results in loss of a functional *Mixta* protein. **d**, Sequences from empty donor sites from five independent germinal *Mixta* revertants¹⁸. The sequence of the wild-type locus in the region of the Tam4 insertion site was obtained from a clone from an *Antirrhinum* genomic library in λ EMBL4. The sequence at the site of Tam4 insertion (filled arrowhead) is labelled *mixta*. The sequences to the right of Tam4 were determined from the 9-kb *Eco*RI fragment (clone 208) isolated¹⁷ using Tam4 as a probe. The sequences to the



left and the 3-bp direct duplication (TTA) were deduced from the wild-type sequence and the size of the direct duplication generated by Tam4 (ref. 6) as only one side of the Tam4 insertion was cloned from the *mixta* line. The sequence footprints generated in the revertants by Tam4 excision are indicated below the line and the remains of the direct duplication are boxed. **e**, The expression pattern of *mixta* shown by cDNA hybridization to RNA from lobes and tubes of corolla, young (buds less than 5 mm long) and older (buds 20–25 mm long) corollas and leaf, seed pod and calyx. *Mixta* expression was restricted to the lobes of older flower corollas.

absent from several independent *Mixta* revertants (Fig. 2a). Cloning of an *Eco*RI restriction-enzyme-digested fragment unique to the *mixta* lines provided a probe from the sequences immediately flanking this Tam4 insertion. The probe hybridized to a unique DNA fragment which shifted in size according to the presence of Tam4 in *mixta* plants and with its absence from revertant plants (Fig. 2b) and established that the flanking DNA was at the *mixta* locus. This probe was used to isolate the wild-type *mixta* genomic sequences and two full-length *mixta* complementary DNA clones (from wild-type petal lobes). Amplification by polymerase chain reaction (PCR) of genomic DNA from five more independent revertants revealed transposon footprints at the site of excision of Tam4 in each revertant line (Fig. 2d).

The amino-acid sequence derived from the full-length *mixta* cDNA clones resembled several Myb-related transcription factors from animals and plants^{7,8}. The strong sequence similarity within the Myb DNA-binding domain (42.7% identity with human c-Myb) and the presence of a sequence capable of forming an amphipathic α -helix in the C-terminal domain (Fig. 3) suggested that the *Mixta* protein is a transcriptional activator.

FIG. 3 Sequence of *mixta* cDNA and its deduced amino-acid sequence. The two repeats of the Myb-related DNA-binding domain are boxed. The region most likely to form amphipathic α -helix is underlined with a dashed line. The conserved positions of the two introns (determined from comparison of the sequences of cDNA and genomic clones) are indicated by arrowheads. Dots indicate every tenth nucleotide. This sequence has been entered into the EMBL Data Library under accession no. X79108.

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   M V R S P C C D K V G V K K G P M -
61  GGAGCTGTAGACGAAGATCAAAAGCTGTGGCTTACATTTAAGAAACATGCTGGAAGCT  120
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121 GGGCTTCCTCCCTTAAAGCTGGCTGAGAGATGTGGAAGAAAGCTGCAATTAAGCT  180
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   I I Q L H A L L G N R W S A I A S H L P -
301 CAAAGAGAACTGACATGAGATCAGAACTCTGGACACATCTTAAAGAGAGCTTTAA  360
   K R T D N E I K N Y W N T H L K K R J L T -
361 CAAGAATGGGAATTGATCCAGTGACCCATAAGCCCAACATCATCTCCGCGCCACG  420
   R M G I D P V T H K P H T H N I L G H G -
421 GACAACATGAAGGATGTGCAACCTTAACACATCGCTCAATGGGAGAGCGCTCGCCTAC  480
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   F V E G I T D I S S S N I V L G A G V L -
841 TACCTAGTAACCTGATAATGTTGTTGATATTTTGAAGAGAACTGGAGCAGTGTCTGA  900
   P S N S D N V V G Y F E E N W S S V L N -
901 ATAATGTGGCTCGTCAATGGATTCGCCAGATGTTTGTAGTAAACGCTAGCTCTT  960
   N V A S S S S M S P D V L V N A S S S -
961 CCTACAAATGTATTAATAGTAGTATTTACGTATTAGAAAAAGCTGTATTAATAGTGA  1020
   Y K M Y *
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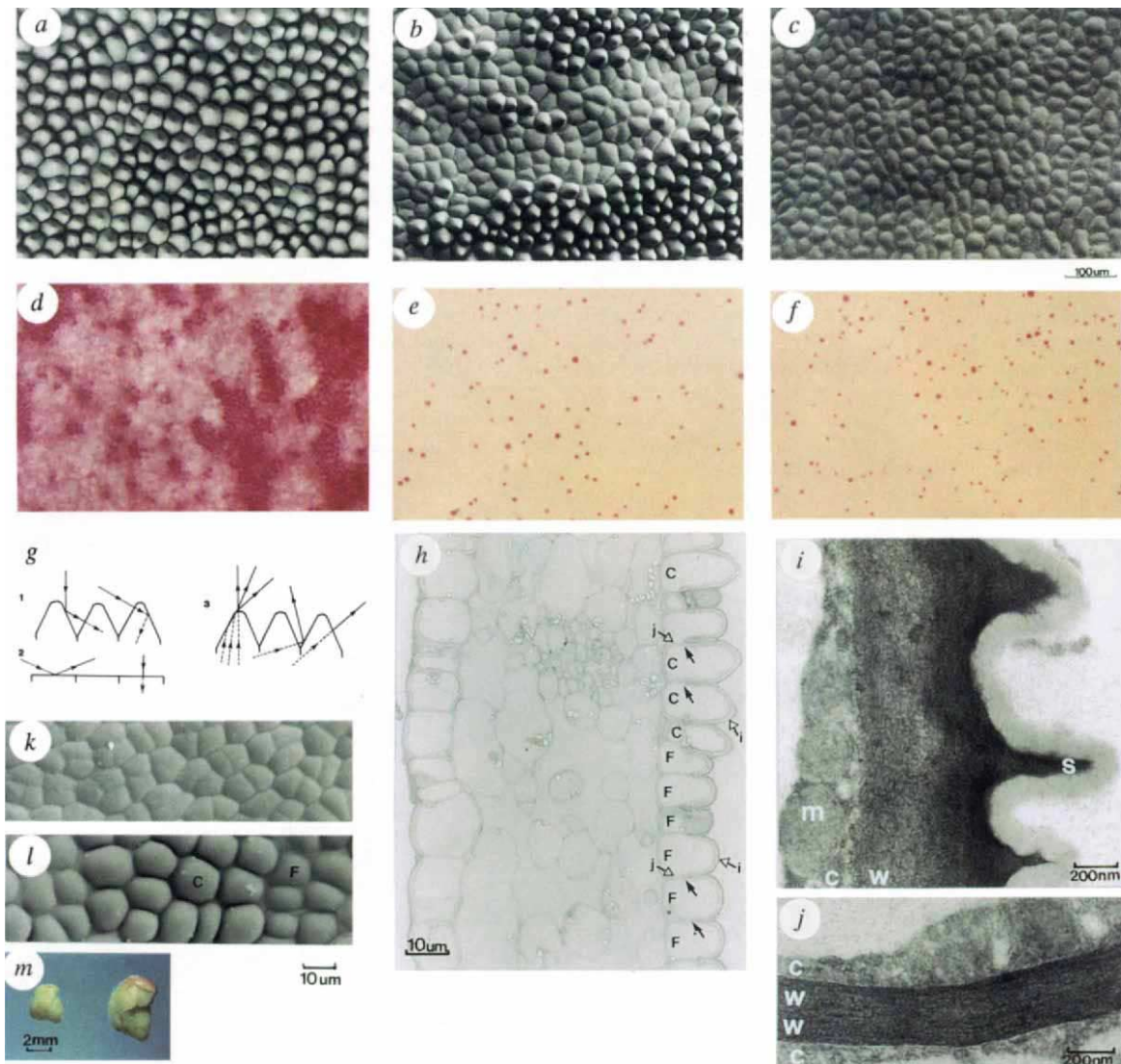


FIG. 4 Effects of *mixta* mutation on epidermal cell differentiation and pigment intensity. *a*, Scanning electron micrograph (SEM) of conical-papillate cells from the inner epidermis of wild-type corolla lobes. The cells are conical, pentagonal at their base (in contrast to the normal amoeboid shape of epidermal cells), and covered with a striated cuticle. *b*, SEM of inner epidermis of lobes from a somatically unstable *mixta* line showing flattened mutant cells, which retain their pentagonal base and cuticular striations, surrounding islands of conical-papillate revertant cells. *c*, SEM of somatically stable *mixta* line showing only flat cells, which retain their pentagonal shape and cuticular striations. *d*, View of pigment in inner epidermis of a somatically unstable *mixta* line showing the clear difference in pigmentation intensity between the paler mutant cells and the darker revertant cells. *e*, Protoplasts¹⁹ made from an epidermal peel of the inner epidermis of wild-type corollas. *f*, Protoplasts made from an epidermal peel of the inner epidermis of stable corollas. No difference in pigmentation intensity could be detected between cells shown in *e* and *f* after removal of cell walls. *g*, Role of epidermal cell differentiation in light absorption and reflection^{3,4}. (1) In a conical epidermis, incident light is reflected by the angled cell walls into the cells, including light arriving at a low angle of incidence, whereas on flat cells (2) such light is reflected off the cell walls. Therefore conical cells enhance the relative amount of light absorbed by the epidermal cells and reduce the amount of reflected white light to enhance the intensity of pigmentation^{3,4}. (3) Light reflected out from the mesophyll below the epidermis will be reflected in many directions by the angled walls of conical-papillate cells, giving the epidermis a bright appearance when viewed from a variety of angles^{3,4}. *h*, Light

micrograph of transverse section²⁰ through the petal lobe of an unstable *mixta* line. Conical revertant cells (C) are seen amid the flattened *mixta* cells (F). The vacuolar size does not change significantly but the surface-area-to-volume ratio of conical cells is estimated to be 0.52 as compared with 0.41 for flat cells. This difference can be observed structurally in the greater amount of thickened wall material and cuticle (labelled (i) here and shown in detail in panel *i*) around the conical cells (the start of the thickened wall is indicated by solid arrows) compared to the more limited amount around the flat cells (shown in panel *j*). Transmission electron micrograph²⁰ of specialized thickened cell wall with striated cuticle, located on the outer surface of the epidermal cells. This wall is ~4-fold thicker than the wall between the cells (panel *j*) and its location is indicated in panel *h* by arrows labelled (i). We estimate this specialized wall to cover, on average, 660 μm^2 per cell in conical cells and 310 μm^2 in flat cells. *j*, Transmission electron micrograph of two appressed walls between epidermal cells (at their base). The wall here is considerably thinner than the outer wall (shown in panel *i*). Its location is indicated by the arrows labelled (j) in panel *h*. In *i* and *j*: c, cytoplasm; w, cell wall; m, mitochondrion; s, striation. *k*, SEM of epidermal lobe cells of 4.5-mm buds of somatically unstable *mixta* line. The cells appear uniformly flat and are still dividing. *l*, SEM of 6-mm buds of somatically unstable *mixta* line. The development of sectors of wild-type conical cells (C) is apparent amid flat cells (F). *m*, The time of development of the conical cells is therefore in buds between 4.5 mm (left) and 6 mm (right) in length, a time that corresponds to the end of the period of rapid cell division and the start of cell expansion in petals²¹, but which is later than the earliest production of anthocyanin in the petal lobes.

Analysis of the *mixta* transcript in the mutant line (Fig. 2c) led us to conclude that insertion of the transposon in the *mixta* line results in the loss of a functional Mixta protein⁹.

The similarity of the Mixta protein to the regulatory products encoded by the *c1* and *p* loci of maize^{10,11} suggested that Mixta could affect floral pigment intensity by activating expression of anthocyanin biosynthetic genes. But no quantitative difference could be measured either in the amount or in the absorption spectrum of the anthocyanin pigment extracted from *mixta* and wild-type lobes.

In contrast, scanning electron microscopy revealed a significant difference between petals from *mixta* and wild-type flowers. The cells of the inner epidermis, which are conical-papillate in wild-type flowers, were altered in the *mixta* mutants (Fig. 4a, c). The change in shape was seen clearly in lines with lower somatic excision frequencies, where no conical cells were observed (Fig. 4c). In lines with high somatic excision, islands of revertant conical cells were seen in a background of flat mutant cells (Fig. 4b). The primary effect of the *mixta* mutation was therefore on the specialized differentiation of the inner epidermal cells of the corolla. To our knowledge, this represents the first time this gene has been shown to affect cell shape. The influence of *mixta* on floral pigmentation intensity appeared to arise from failure to develop the conical cell form (Fig. 4g). The angled walls of conical-papillate cells reflect light through the epidermis, especially incident light from low angles³ (Fig. 4g, 1), whereas flat cells reflect it back³ (Fig. 4g, 2). Convergent walls of papillate cells also scatter light emerging by reflection from the underlying mesophyll, so that petal brightness remains constant regardless of the angle of observation^{3,4} (Fig. 4g, 3).

We confirmed that *mixta* affected pigment intensity through changing cell shape by making protoplasts from strips of the inner epidermis of corolla lobes from flowers of *mixta* and wild-type lines. Whereas the difference in cellular pigmentation intensity was clear in the context of the specialized cell structure in the inner epidermis (Fig. 4d), removal of the cell walls resulted in *mixta* and wild-type protoplasts with identical colour intensity (Fig. 4e, f).

The inner epidermal cells of the corolla lobes develop a pentagonal base, as well as being papillate, and they are covered by specialized striations of cuticular wall material (Fig. 4a). In the *mixta* mutant, the inner epidermal cells remain pentagonal and retain their striations (Fig. 4b, c). It is therefore likely that gene expression controlled by *mixta* is part of the machinery required for the synthesis of the specialized conical-papillate structure, rather than that determining epidermal cell fate, as other features of cellular differentiation remain in the mutant.

Sections of petal tissue revealed that the most significant cellular feature in conical-papillate cells was the thickened cell wall and striated cuticle, which developed only on the outer surface of the cells (Fig. 4h, i) and was fourfold thicker than the wall at the base of the epidermis and between the cells (Fig. 4h, j). Sections from unstable *mixta* lines (Fig. 4h) showed that there was much more specialized thickened wall in conical *Mixta* revertant cells (mean estimate ~660 μm^2 per cell) than in flat *mixta* cells (mean estimate ~310 μm^2 per cell) and although the surface area of flat cells was significantly less than that of conical cells, the volume was not. These sections suggest that *mixta* controls cell shape by activating the directional synthesis of specialized epidermal cell-wall material. Another Myb-related transcription factor (Myb308; ref. 8) can change the shape of leaf palisade cells through modification of cell-wall production (C.M., manuscript in preparation), implying that several plant *myb* genes may be involved in this cellular function. The *myb*-related gene *gl1* (refs 12, 13), which is required for trichome development in *Arabidopsis*, might have a similar role, although recent data suggest that *gl1* is required for an early endoreduplication in trichome development¹⁴ rather than for directly controlling cell shape. Analogous experiments with unstable *mixta* lines showed that *mixta* does not affect the cellular DNA con-

tent. Sequence comparisons do not show a structurally related subgroup among plant Myb proteins affecting cell shape.

Inactivation of *mixta* demonstrates the degree to which cell form can influence pigment intensity in flowers. Conversely, pigment intensity can now be used as a simple screen for epidermal cell-shape mutants. Wild-type petals have a velvet sheen, whereas the flattened cells of *mixta* mutants are dull. Insects can also distinguish between the epidermal cells of different flowers through feeling their shape¹⁵, which reinforces the importance of *mixta* in the attraction of pollinators. *Mixta*, with its role in cellular differentiation, is also a strong candidate for a target gene activated relatively late (Fig. 2e and Fig. 4k, l and m) by the homeotic genes that determine petal identity in *Antirrhinum* (*deficiens* and *globosa*¹⁶). It therefore represents a lower level in the hierarchy of transcriptional control operating between organ determination and cellular differentiation in the process of floral morphogenesis. □

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Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in *Drosophila*

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MUCH of our present knowledge of the biological processes involved in pattern formation in *Drosophila* is derived from segmentation analysis^{1,2}. Comparatively little is known about the genetic requirement and mechanisms underlying the formation and separation of germ layers by morphogenetic movements during

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gastrulation³. Here we show that the *Drosophila* gene *huckebein* (*hkb*), a member of the gap-gene class of segmentation genes^{4,5}, is required for germ-layer formation at blastoderm. Absence of the *hkb* product, an Sp1/egr-like zinc-finger protein, causes the ectodermal and mesodermal primordia to expand at the expense of endoderm anlagen. Conversely, ectopic expression of *hkb* inhibits the formation of the major gastrulation fold which gives rise to the mesoderm and prevents normal segmentation in the ectoderm. Thus, *hkb* is necessary for endoderm development and its activity defines spatial limits within the blastoderm embryo in which the germ layers are established.

Germ-layer formation in *Drosophila* begins by two major gastrulation movements once the homogeneous sheet of cellular blastoderm is established⁶. Ectoderm and mesoderm separate by the formation of the ventral furrow, a row of cells covering the ventral-most position of the embryo (Fig. 1a, c). The endoderm anlagen invaginate from two separated blastoderm regions which are anterior to the ventral furrow⁷ and at the posterior pole⁸, respectively (Fig. 1e). The developing anterior and posterior endoderms grow towards the centre, where they combine to form the midgut (Fig. 1g), the only endodermal derivative of the fly (reviewed in ref. 9). The cells that remain at the surface of the gastrulating embryo develop mainly epidermis and neural tissue⁶. Factors necessary for the establishment of mesodermal anlagen have been identified¹⁰, yet the genetic requirements for endoderm formation and for subdividing the cellular blastoderm into germ-layer anlagen remain unknown. Here we show that the early zygotic activity of the gene *hkb* is essential for endoderm specification and that its expression is incompatible with normal mesodermal and ectodermal development.

hkb is a terminal gap gene of *Drosophila*^{4,5}. Genetic analysis indicated that the combined activities of *tailless* (*tll*)¹¹ and *hkb* are specifically involved in mediating the maternal terminal

information of the *torso* signalling pathway at the posterior end of the blastoderm embryo⁴. In the absence of the two terminal gap-gene activities, as in *hkb,tll* double-mutant embryos, the expression domains of the 'central gap genes' expand posteriorly⁵ and the trunk anlagen, as defined by the stripe expression of pair-rule genes, are shifted into the posterior pole region of the embryo⁴. These results suggested that both *hkb* and *tll* are specific factors for distinguishing the segmented trunk from the non-segmented tail region of the embryo, possibly by local suppression of central gap genes in the blastoderm pole regions⁵.

The phenotypic consequences of absent *hkb* activity can be detected as early as the beginning of gastrulation. Both by morphological criteria (Fig. 1a, b) and specific marker gene expression (Fig. 1c, d), *hkb* mutant embryos show an abnormal expansion of the ventral furrow towards the pole regions in *hkb* mutant embryos (Fig. 1b, d). This indicates that *hkb* activity is required spatially to restrict the limits of the ventral furrow to the central portion of the gastrulating wild-type embryo. The formation of the 'polar plate', which normally carries the combined anlagen of the posterior endoderm and the ectodermal hindgut^{6,8}, and the subsequent amnioproctodeal invagination are indistinguishable from wild-type embryos. To determine whether the amnioproctodeal invagination of *hkb* mutant embryos carries both the ectodermal hindgut and endodermal midgut anlagen, we analysed the expression of the endodermal markers PS11 (ref. 12) and *prospero*¹³. PS11 (Fig. 1e) expression labels the expanding endoderm anlagen and continues to label the midgut throughout embryonic development (Fig. 1g); *prospero* labels adult midgut precursor cells, a subset of the embryonic midgut endoderm¹³ (Fig. 1i). *hkb* mutant embryos fail to express PS11 (Fig. 1f, h) and *prospero* expression is absent in the region where the endoderm anlagen normally develop (Fig. 1j). Mor-

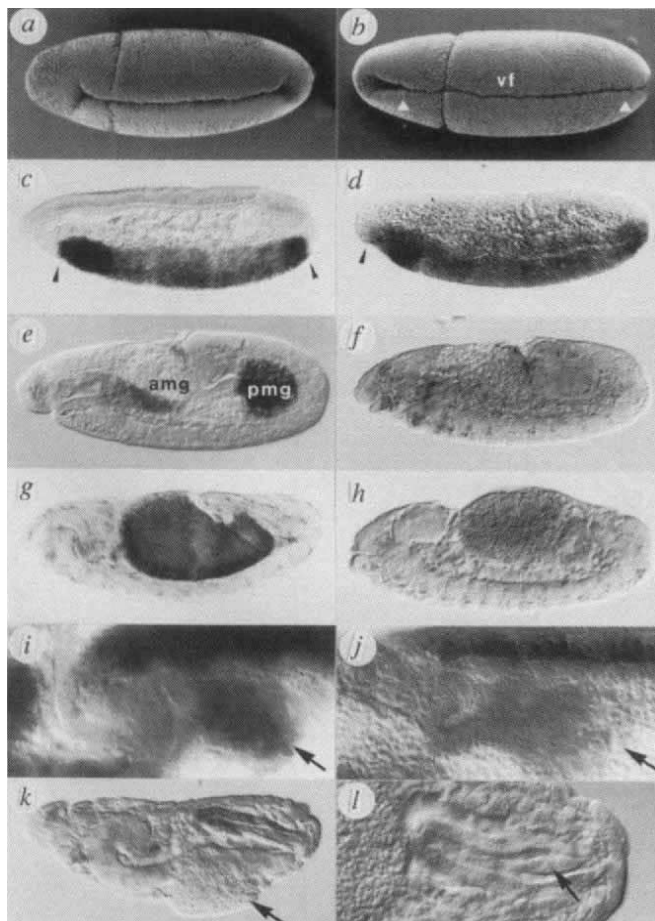


FIG. 1 Lack of *hkb* activity causes expansion of the ventral furrow and absence of the endoderm. a, Scanning electron micrograph of a gastrulating *Drosophila* wild-type embryo (ventral view). b, Corresponding view of an *hkb* mutant embryo showing that the ventral furrow (vf) expands towards the termini of the embryo (size of wild-type vf is shown between arrowheads). c and d, *zfh1* expression in wild-type (c) and *hkb* mutant (d) embryos. *zfh1* serves as a molecular marker for the blastoderm cells that eventually form the ventral furrow²¹; note the expanded *zfh1* expression in the *hkb* mutant. The anterior and posterior borders of the ventral furrow (arrowheads) correspond in position to the sites of invagination of the combined endodermal and ectodermal gut anlagen⁶. e and f, PS11 expression in the developing endodermal midgut of wild-type (e) and *hkb* mutant (f) embryos. PS11 expression serves as an early molecular marker for endoderm development¹². amg, Anterior midgut; pmg, posterior midgut. Note the lack of PS11 expression in the *hkb* mutant embryo. (g and h), PS11 expression in late wild-type (g) and *hkb* mutant (h) embryos. Note that PS11 expression, which is maintained throughout endoderm development¹², labels the midgut of wild-type embryos and that *hkb* mutants lack the midgut. Orientation of embryos is anterior left and dorsal up (note ventral view in a, b). i and j, *prospero* expression¹³ in a subset of midgut endoderm in wild-type (i) and *hkb* mutant (j) embryos. Those cells are precursors of the adult midgut¹³ in wild-type embryos (arrow in i). Note the lack of *prospero* expression in *hkb* mutant embryos (arrow in j). k, In the absence of the midgut, the yolk fails to be engulfed and distributes in ectopic locations (arrow). l, Enlarged posterior region of a *hkb* mutant embryo indicating that the hindgut (see also i) is a closed tube (arrowhead) which contains the germ cells that normally would have left through endodermal tissue to reach their final position in a pair of mesodermal pockets representing the developing gonadal tissue at the corresponding stage of a wild-type embryo.

METHODS. Scanning electron microscopy on dechorionated and devitellinized embryos was performed as described²². Photographs were taken with a Cambridge Stereoscan 150 electron microscope. Whole-mount *in situ* hybridization of embryos using the *zfh1* cDNA probe²¹ and antibody detection of *prospero* and β -galactosidase expression (in the PS11 enhancer trap line) have been described^{12,13,21}.

phological inspection of differentiated *hkb* embryos indicates that the endodermal midgut is missing (Fig. 1*k, l*). Consequently, the yolk of *hkb* mutant embryos distributes into ectopic positions of the embryo as it is not surrounded by the midgut (Fig. 1*k*). The remaining ectodermal hindgut of *hkb* mutant embryos forms a closed tube, trapping most of the germ cells which would normally migrate through the endodermal midgut portion into the gonadal mesoderm⁹ (Fig. 1*l*). These results indicate that blastodermal *hkb* activity is not only required to suppress the action of the trunk segmentation genes^{4,5}, *hkb* activity is also essential in specifying endodermal cell fate and spatially restricts ventral furrow formation at both ends of the blastoderm embryo. As the ventral furrow carries the anlagen of the meso-

derm, *hkb* is required for the normal formation of the three germ layers in *Drosophila*.

To determine the nature of the *hkb* gene product and to characterize *hkb* further in a gain-of-function situation (see below), we isolated *hkb* DNA by positional cloning (Fig. 2*a, b*). The *hkb* transcript (Fig. 2*c*) encodes a Sp1/egr-related zinc-finger protein (Fig. 3*a*). It contains a glutamine-rich region corresponding to an activation domain¹⁴ and an alanine-rich region similar to repressor domains of *Drosophila* transcription factors¹⁵. Based on these similarities (Fig. 3*b, c*), the *hkb* protein can be tentatively grouped as a DNA-binding transcriptional regulator.

Expression of *hkb* was first detected in two symmetrical polar caps in the terminal regions of the syncytial blastoderm (Fig.

FIG. 2 Localization, cloning and expression of the *hkb* gene. *a*, Cytogenetic localization of the *hkb* locus. *hkb* is included in the synthetic deletion *Df(3R)P30-XM14^R* (uncovers the cytogenetic interval 82A2-6 on the right arm of the third chromosome) by recombination of inversion chromosomes *In(3R)P30* and *In(3R)XM14*. *In(3R)XM14* develops a weak *hkb* phenotype (B.C., unpublished result). Thus, *hkb* is likely to localize near the inversion breakpoint. To clone the gene, we obtained a P-element, line A321.3M3 (ref. 12), inserted into the 82A region. Genomic sequences flanking the P-element insertion site were cloned to initiate a chromosomal walk which covers ~200 kb of DNA of the 82A3-6 region that includes the *In(3R)XM14* breakpoint. Overlapping cosmids and λ -phage DNA fragments encompassing the cloned region were tested for the presence of genes expressed in the early embryo by whole-mount *in situ* hybridization. DNA of one *EcoRI* fragment of the C370 cosmid clone hybridized to a single transcript (c) expressed in the terminal regions of the blastoderm embryo (d). *b*, The terminally expressed transcript (arrow, 3' direction) was mapped to an internal *AccI*-*Clal* fragment of the C370 cosmid DNA (abbreviations: A, *AccI*; Bg, *BglII*; C, *Clal*; M, *MluI*; RI, *EcoRI*; RV, *EcoRV*; Sm, *SmaI*). After P-element-mediated transformation, a 14-kb transgene (black bar), which codes for a single 1.8-kb transcript (c), provided significant phenotypic rescue to transheterozygous *hkb¹/hkb²* embryos⁴; about 4% of transgene containing transheterozygous *hkb¹/hkb²* embryos survived to adulthood. The adult flies do not fly and fail to respond to optic stimuli (G.B. and H.J., unpublished results). Transgene-containing *hkb* mutant embryos develop a normal embryonic midgut and a normally sized ventral furrow; head involution is normal, although labral derivatives²³ are often abnormal (data not shown). We also analysed mutation-associated lesions of *hkb* mutant alleles; the *hkb¹* allele is a small deletion within the transcript (positions 303–616; Fig. 3); the *hkb^{XM9}* allele is a 1.7-kb deficiency in the 5' region of the transcript, removing most of the coding region (hatched bars). These results and the transgenic rescue establish the identity of the *hkb* gene. *c*, Northern blot of poly(A)⁺ RNA from 0–4-h-old embryos showing a 1.8-kb transcript; arrows indicate the position of 1.4- and 2.4-kb marker bands. *d*, Whole-mount *in situ* hybridization of a syncytial blastoderm using the *EcoRI* fragment of the C370 cosmid DNA (*b*). *e* and *f*, Enlarged anterior (*e*) and posterior (*f*) region during the early phase of gastrulation. *e*, The anterior *hkb* expression domain has shifted ventrally, bordering the anterior margin of the ventral furrow (arrowhead). *f*, The posterior domain (covering the combined anlagen of ectodermal hindgut and posterior endoderm; above arrowhead) borders the posterior margin of the ventral furrow. *g*, During gastrulation, *hkb* transcripts vanish in the anterior and posterior terminal regions. *De novo* expression of *hkb* in a metamereric pattern is found in the developing central nervous system and in the salivary gland placodes. *h*, *hkb* expression remains in the central nervous system throughout embryonic development. *hkb* functions in salivary gland placodes and in the central nervous system will be presented elsewhere (Q.C.-L. et al., unpublished results).

METHODS. Cloning, chromosomal walking and *in situ* hybridization to whole-mount embryos using digoxigenin-labelled DNA as probe have been described²⁴. *hkb* cDNA clones were isolated from a 0–4 h embryonic cDNA library³⁰. The extent of the coding region was determined by hybridization of the cDNA clone to genomic DNA, by primer extension analysis (data not shown), and by DNA sequencing of both genomic and several cDNAs. Germ-line-dependent P-element-mediated transformation of the 14-kb transgene was performed with the Carnegie P20 vector²⁵. For sequencing, DNA of wild-type and of selected homozygous *hkb* mutant embryos was used for PCR to amplify the *hkb* coding region; several independently obtained clones were sequenced for each allele as described²⁴.

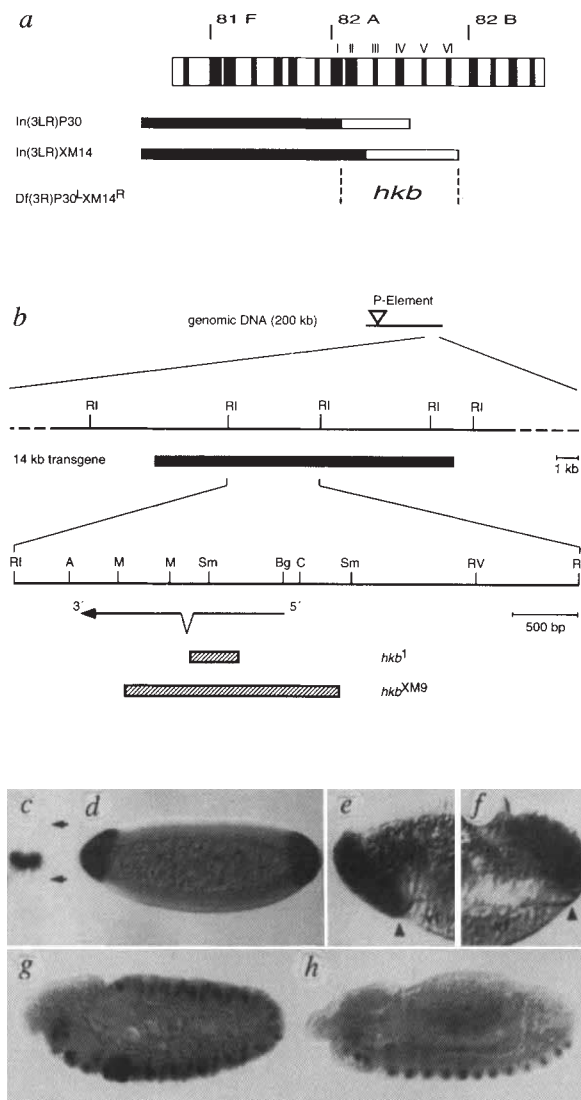
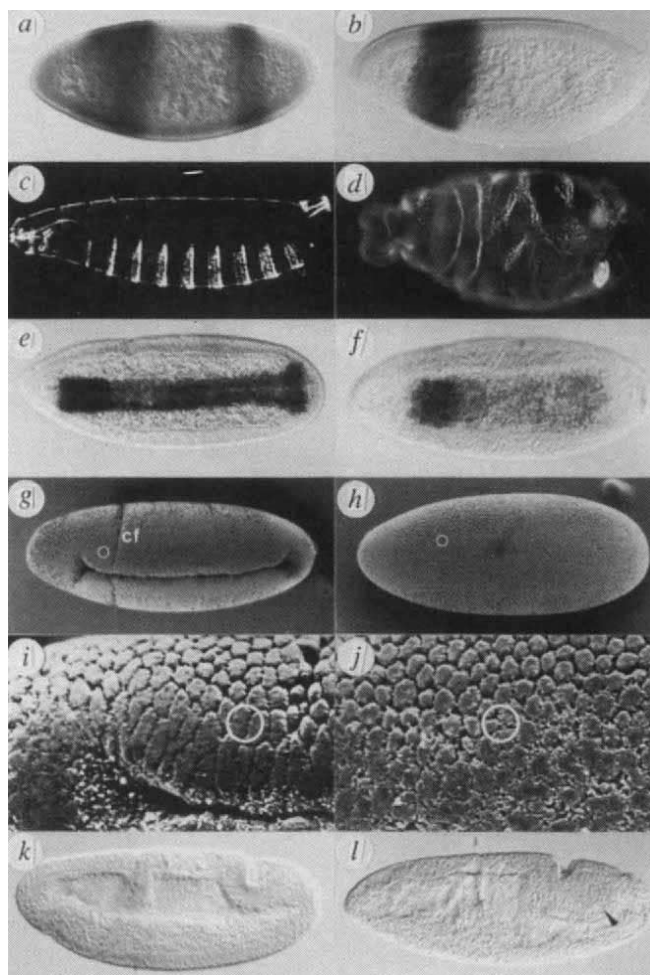


FIG. 4 Ectopic expression of *hkb* causes defects in the ectodermal anlagen and prevents invagination of the ventral furrow. *a*, Expression of the gap gene *giant*²⁸ in a wild-type blastoderm. *b*, Ectopic *hkb* expression at blastoderm stage causes the absence of the posterior *giant* expression domain. In less extreme cases (not shown), the posterior *giant* expression domain is significantly reduced in comparison to the anterior expression domain. *c* and *d*, Cuticle preparation of wild-type larva (*c*: anterior left, dorsal up) and larva which has received ectopic *hkb* expression at blastoderm stage (*d*). Ectopic *hkb* expression causes the disturbance of the segmentation pattern, consistent with its interference with the activity of the *Drosophila* segmentation gene cascade (*b*). Note that these embryos are shorter than the wild-type like embryos mutant for any of the central gap genes. *e*, *zfh1* expression²¹ in wild-type embryo. *f*, The level of *zfh1* expression is reduced, indicating that ectopic *hkb* activity is acting in the cells that give rise to the ventral furrow in wild type (Fig. 1). Note that the reduced level of *zfh1* expression continues to cover the region of initial *zfh1* expression at a stage when most *zfh1*-expressing cells would be part of the invaginating ventral furrow, implying that the invagination does not occur. *g* and *h*, Scanning electron microscopographs showing ventral furrow formation in wild-type embryos (*g*) and the absence of a ventral furrow in embryos in which *hkb* was ectopically expressed (*h*). Although a ventral furrow does not form in embryos after ectopic *hkb* expression, the mesodermal cells that normally invaginate to form the ventral furrow (coinciding with the *zfh1* expression domain; see *f*) can be morphologically distinguished from normal ectodermal cells; see enlarged mesoderm/ectoderm boundaries in *i* and *j*. White circles indicate the same position in embryos in *g* and *i*, and in *h* and *j*, respectively. Note that ectodermal cells do not seem to be affected by ectopic *hkb* expression. *k*, Optical section through wild-type embryo. *l*, Corresponding view of an embryo after ectopic *hkb* expression showing that invagination of the amnioproctodeum (arrow) occurs normally.

METHODS. For ectopic *hkb* expression at blastoderm stage, embryos containing a *hsp-hkb* gene construct see below were heat-shocked for 20 min at 37 °C between 1.0–2.5 h of development. Embryos were fixed and stained 45 min after heat shock. As controls, embryos of the same genotype but lacking the *hsp-hkb* transgene were treated in parallel. *In situ* hybridization to whole-mount embryos was done with cDNA probes of *zfh1* (ref. 21) and the *giant* transcript²⁸. β -Galactosidase expression and SEM are described in Fig. 1 legend. P-element-mediated transformation of flies and subsequent crosses with the *hsp-hkb* transgene construct (which contains the *Smal*/EcoRI fragment of the 3' region of the cDNA fused to the *Bgl*II/*Smal* genomic DNA (Fig. 2) inserted into the P-element transformation vector pCaSpeRhspNot) were done as described²⁹.

transduction pathway². In contrast, its anterior expression is controlled, in addition to *torso* activity, by the combined activities of the anterior and dorsoventral maternal pattern-forming systems (G.B. and H.J., unpublished result). Once activated, terminal *hkb* activity is essential for endodermal development and it locally overrules both ectoderm and mesoderm development that would otherwise occur in the terminal regions of the embryo (Fig. 1*b, d*). When ectopically expressed, *hkb* inhibits ventral furrow formation throughout the embryo, but the am-



nioproctodeal invagination continues to occur normally (Fig. 4*f, h, l*). Thus, the two major morphogenetic movements during *Drosophila* gastrulation, which separate mesoderm and endoderm from ectoderm, are under separate genetic control. Given the generality of these ontogenetic processes¹⁷ and the evolutionary conservation of key factors guiding animal development (for example, see refs 18–20), our results may provide a valuable lead into the genetic requirement that causes separation and differentiation of germ layers. □

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Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice

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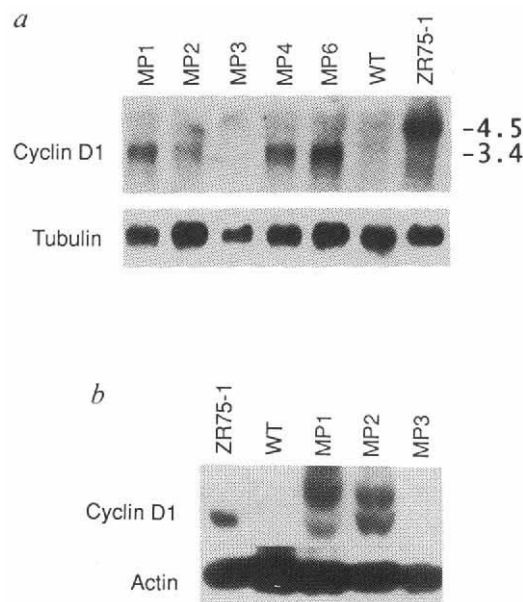
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PHYSICAL associations between cyclins, viral oncogenes and tumour suppressor genes imply a central role for cyclins in growth control^{1,2}. Cyclin D1 was identified as a candidate oncogene (PRAD1) in tumour-specific DNA rearrangements^{3,4} and is suspected to be a contributor to several types of neoplasms including breast cancer^{5,6}. Cyclin D1 also rescues G1 cyclin-defective *Saccharomyces cerevisiae*⁷, and is a growth-regulated gene⁸. Despite evidence suggesting that cyclin D1 is an oncogene, its ability to transform cells directly in culture remains controversial⁹⁻¹⁶. To evaluate its potential to deregulate growth *in vivo* in a physiologically relevant tissue we overexpressed cyclin D1 in mammary cells in transgenic mice. We report here that overexpression of cyclin D1 resulted in abnormal mammary cell proliferation including the development of mammary adenocarcinomas. We conclude that overexpression of cyclin D1 deregulates cell proliferation and can induce tumorigenic changes in mammary tissues, suggesting that cyclin D1 indeed plays an important oncogenic role in breast cancer.

More than 10 different studies report amplifications of DNA on chromosome band 11q13, which includes the gene for cyclin D1, in 15 to 20% of human breast cancers⁶. To assess the role of cyclin D1 as a candidate oncogene, we examined the consequences of overexpression of cyclin D1 in transgenic mice. The cyclin D1 complementary DNA⁴ was cloned into an expression plasmid containing the mouse mammary tumour virus long terminal repeat (MMTV-LTR), plus SV40 intron and polyadenylation signals. A linearized fragment from the MMTV-cyclin D1 construct was microinjected into fertilized mouse oocytes and six transgenic founder mice were identified.

Transgene expression was demonstrated by RNA blots and immunoblots (Fig. 1). The expected 3.4 kilobase (kb) transgenic messenger RNA transcript was detected in mammary tissue from five examined lines (Fig. 1a). No endogenous cyclin D1 expression was detected in RNA isolated from the mammary glands of transgenic or non-transgenic females. Cyclin D1 protein was



migrates at about 4.5 kb as seen in the ZR-75-1 human adenocarcinoma cells; in contrast, the transgene migrates at about 3.4 kb in the mammary epithelium of the transgenic mice corresponding to the expected length of the transgenic fusion construct. Transgenic lines analysed included TG.MP 1 to 4, and MP6 (MP1 to MP4; MP6). All mice were analysed between 2 and 3 weeks of gestation at about 10 weeks of age; mammary tissue was collected at 10 days of gestation to examine transgene expression at maximum levels during pregnancy. No expression of cyclin D1 is seen in the corresponding age and pregnancy-matched wild-type mouse (WT). The last lane includes mRNA from a human breast cancer cell line (ZR-75-1) known to express high levels of cyclin D1. RNA blots containing 5 µg total RNA in each lane were probed sequentially for cyclin D1 and tubulin as indicated. *b*, Analysis of cyclin D1 immunoreactive protein in mammary glands of MMTV-cyclin D1 transgenic mice. Tissue extracts from mammary glands were obtained from 10-week-old, pregnant female transgenic mice as in *a* (MP1, 2, 3) to compare to an age- and pregnancy-matched wild-type mouse (WT). These were further compared to a breast carcinoma cell line (ZR-75-1) that contains an amplified cyclin D1 locus and is known to overexpress p35 cyclin D1. Although ZR-75-1 cells only express a single form of cyclin D1 at 35K, other breast cell lines typically express a 35–37K doublet as seen in the transgenic mice (data not shown). The basis for this difference is unknown. Immunoblots were probed sequentially with monoclonal antibodies specific for cyclin D1 and actin as indicated.

METHODS. *a*, RNA was isolated from the indicated tissues by the guanidine thiocyanate method. Each lane was loaded with 5 µg of total cellular RNA, and Northern blots used standard techniques. Cyclin D1 transcripts were detected with an α -³²P-labelled random-primed probe made from the 1.4 kb EcoRI fragment of λ P1-4 containing the complete human cyclin D1 coding region⁴. *b*, Tissue samples were teased and lysed in SDS sample buffer, then sonicated and heated to 90 °C for 10 min. 60 µg of total protein of each sample was separated on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to Immobilon membranes (Millipore) at 5 V for 12 h at 4 °C. Blocking was in 5% milk, 10 mM Tris pH 7.4, 150 mM NaCl, 0.2% Tween 20 for 1 h. The monoclonal antibody specific for cyclin D1 was produced from BALB/c mice immunized with bacterially expressed cyclin D1 protein (E.L., L.Z., A.A. and E. Harlow, manuscript in preparation); mouse splenocytes were fused with NS1 myeloma cells and hybridomas producing monoclonal antibodies specific for cyclin D1 were identified by ELISA and immunoprecipitations with *in vitro* translated and native cyclin D1. The antibody used (HD11) was specific for cyclin D1 and did not exhibit cross reactivity for cyclins D2 or D3. The antibody is reactive with both mouse and human cyclin D1. For immunoblots the supernatant culture medium was used at a 1:4 dilution. Primary antibodies were used at 1:4 dilution for cyclin D1 monoclonal antibody, and 1:5,000 for monoclonal antibody against actin. Secondary antibodies were anti-mouse immunoglobulins (Cappel; 1:5,000 dilution). The detection system used was chemiluminescence (ECL; Amersham).

FIG. 1 Analysis of cyclin D1 mRNA and protein in mammary epithelium of MMTV-cyclin D1 transgenic mice. *a*, Expression of cyclin D1 mRNA in mammary glands of wild type (WT) and transgenic mice (MP). F₁ offspring from the five founder mice that transmitted the transgene were examined for expression of cyclin D1. Total mRNAs extracted from mammary tissues of transgenic MMTV-cyclin D1 lines were analysed using the human cyclin D1 cDNA probe. Endogenous cyclin D1 mRNA

TABLE 1 Tumour incidence in MMTV-cyclin D1 transgenic strains

Transgenic line	Mice older than one year*	Pathological analysis complete	Mice with mammary tumours	Mice with other tumours	Mean age at onset†
MP1‡	9	9	5	2§	534 ± 35
MP2	5	1	1	0	511
MP6	2	2	2	0	630

Cohorts of mice in each of five MMTV-cyclin D1 transgenic strains have been observed for tumour development. Mammary and other tumours have been observed in three of the strains after a latency longer than 1 year. No mice have yet lived longer than 1 year in the remaining two strains. Consequently, only mice that have survived longer than 1 year in the three strains developing tumours are analysed in this table.

* Eight females and one male mouse were examined in the MP1 strain. The mice in the MP2 and MP6 strains were all female.

† Data reported as mean age in days ± standard error of the mean for MP1 where $N=7$. For MP6 the mean of the ages of two mice is reported.

‡ The total tumour incidence in this strain is 7 of 9 mice with no mice dying of any other causes. Compared to our wild-type cohort (0/15 mice developing tumours) $P \leq 0.000104$ by chi-square analysis using the Fisher exact test.

§ The two additional tumours in this strain include a uterine wall sarcoma metastatic to the liver and a testicular tumour also metastatic to the liver.

overexpressed in transgenic mammary tissues compared to wild-type mice and the levels of overexpression were comparable to those seen in a human breast cancer cell line (ZR-75-1) containing amplified and overexpressed cyclin D1 (Fig. 1b)⁵.

The MMTV-cyclin D1 transgenic females developed proliferative disturbances of their mammary tissue that correlated with the age of the animal, the number of pregnancies, and the level of expression of the transgene. We examined mammary tissues every 2 months over the first 10 months of their life span. Evidence of proliferative abnormalities coincided with the earliest expression of the transgene after sexual maturity was reached and showed progressive increases with ageing. Whole-mount preparations revealed significant lobulo-alveolar development with extensive side bud formation off the primary ducts in cyclin D1 transgenic mammary glands compared to non-transgenic wild types (data not shown). Histological examination of transgenic mammary glands confirmed the significance of these whole-mount abnormalities (Fig. 2). Numerous primary side buds and

extensive lobulo-alveolar development were identified in several different transgenic lines at several ages.

Both mammary adenocarcinomas and other tumours developed in cohorts of MP1, MP2 and MP6 mice after 1 year of life (Fig. 3; Table 1). Nine MP1 mice (eight females and one male) have been observed for longer than 1 year of age. Five females developed mammary tumours during that time, and four of the mice developed multiple independent tumours. The tumours included mammary adenocarcinomas with papillary and cribriform elements, and mammary adenocarcinomas with squamous differentiation. Additional tumours in MP1 mice included a testicular sarcoma with extensive metastases to the liver and a spindle cell sarcoma of the uterine wall, also metastatic to the liver. In a second strain (MP6), we evaluated two females older than 1 year. Both of these mice developed mammary adenocarcinomas with a papillary pattern. In the third strain of mice demonstrating tumours, five mice have been observed longer than 1 year and one mouse has developed a mammary adenocarcinoma. The incidence of mammary tumour formation for the MP1, MP2 and MP6 mice that have lived long enough fully to assess tumour formation therefore totals 8/12 mice with a mean age of onset of 551 ± 26 days. The remaining four mice in the MP2 strain are still younger than the mean age at which tumours occurred in the MP1 and MP6 strains and may yet develop tumours.

The pattern of occurrence of tumours and mammary hyperplasia in our transgenic strains strongly supports the conclusion that these lesions resulted from the effects of the transgene. Hyperplastic changes occurred in all of the transgenic lines. Occurrence of tumours in three independent transgenic lines (MP1, MP2 and MP6) further demonstrates that tumour formation was not the result of insertion effects of the transgene. In addition, no spontaneous mammary adenocarcinomas developed in any of three cohorts of wild-type, inbred FVB mice maintained in parallel with these experiments (each cohort containing 15 mice). The absence of mammary tumours in wild-type mice of the FVB strain has been further confirmed in a wide experience with this line¹⁷. Furthermore, multiple independent mammary adenocarcinomas developed in the MMTV-cyclin D1 transgenics in striking contrast to spontaneous adenocarcinomas in normal mice

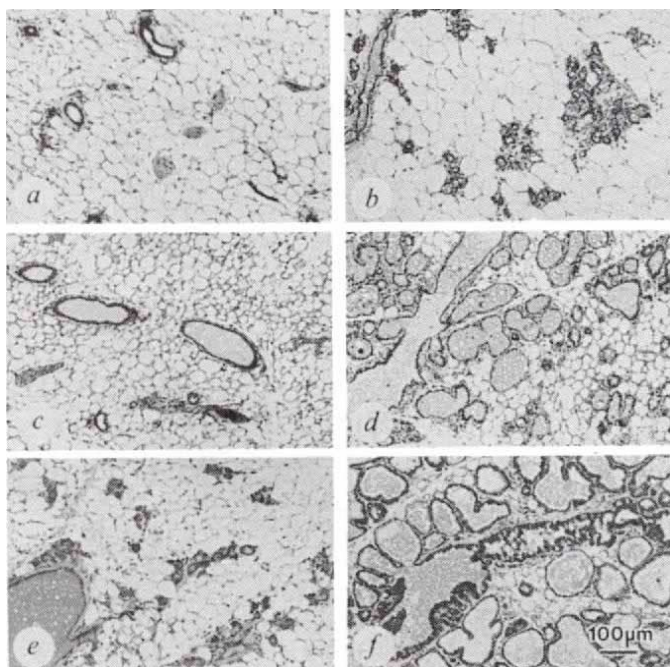
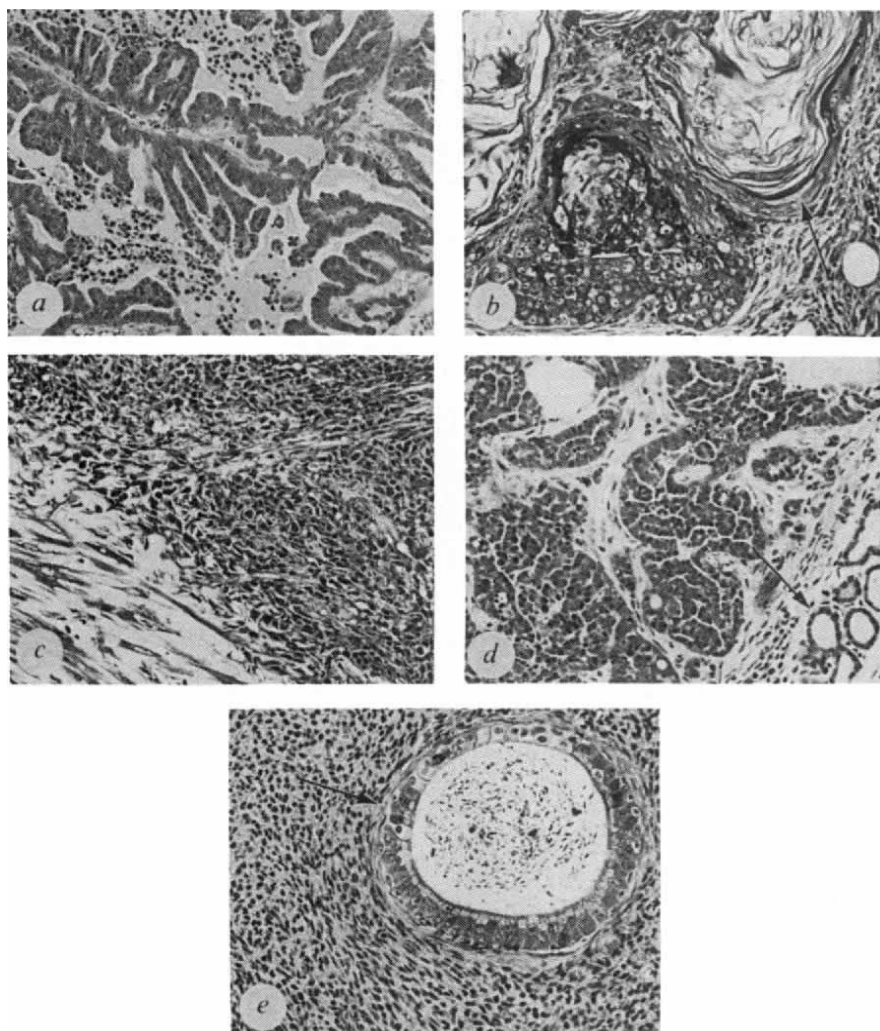


FIG. 2 Photomicrographs of age-matched wild-type (a, c and e) and transgenic mammary glands (b, d and f). The different transgenic strains illustrated include TG.MP1 (b), TG.MP3 (d) and TG.MP2 (f). Young nulliparous MP-1 females first demonstrated significant hyperplasia at 2 months of age (b) compared with age-matched nulliparous non-transgenic animals (a). Older, 4-month-old transgenic females from additional lines of mice had even more dramatic proliferative changes (d, TG.MP3) compared with age-matched wild-type mice (c). Finally, the mammary glands of multiparous MP2 animals, 6 months post-weaning, had extensive alveolar development with papillary epithelium lined by cells with hyperchromatic, slightly pleomorphic nuclei (f) which was not observed in age- and pregnancy-matched wild-type mice (e). Even in mice with a relatively mild phenotype (MP3), the mammary epithelium contained mitotic figures and actively secreting cells (d). Comparisons of all sets illustrate extensive lobuloalveolar development in the transgenic mice which is not seen in the wild-type mice. In particular, note the atypical hyperplasia in the main duct of the transgenic mammary gland after weaning (f). Scale bar (f), 100 μ m. Standard 10- μ m paraffin sections were stained with haematoxylin and eosin.

FIG. 3 Photomicrographic montage illustrating haematoxylin and eosin stained tumours found in transgenic cyclin D1 mice ($\times 220$). *a*, A papillary mammary adenocarcinoma from an MP1 female. *b*, An area from mammary tumour in the same animal illustrating squamous differentiation in an adenoacanthoma. Note the extensive laminated keratinaceous debris in the upper half of the photo (arrow). *c*, A uterine sarcoma from the same transgenic line (MP1) showing the densely crowded spindle cell component. This tumour resulted in extensive metastases to the liver (not shown). *d*, A poorly differentiated mammary adenocarcinoma with a papillary pattern in a different transgenic line (MP6). Note the adjacent non-neoplastic mammary glands in the lower right-hand corner (arrow). *e*, An extensive testicular sarcoma (MP1) surrounding epididymal tissue. Development of testicular tumours is consistent with the usual pattern of expression of MMTV-driven transgenes in male mice. Standard 10- μ m paraffin sections were stained with haematoxylin and eosin.



which are rarely multifocal. Finally, immunohistochemical staining of all of the tumours tested, including the testicular and uterine tumours, demonstrated high-level cyclin D1 expression in tumour tissues (data not shown).

Our results add weight to the candidacy of cyclin D1 as a key breast oncogene on the 11q13 amplicon. Although the 11q13 band contains additional genes¹⁸⁻²², cyclin D1 is consistently amplified and overexpressed in tumours containing the 11q13 amplicon^{5,23}. Amplification of 11q13 is correlated with frequent distant metastases, the presence of oestrogen receptors, and a

shorter disease-free survival in node-negative patients²⁴. Furthermore, the genetic events that produce isolated hyperplastic changes in breast are not well understood, although these pre-neoplastic lesions are increasingly recognized as an important clinical problem²⁵. Thus, our data are consistent with the formulation that cyclin D1 overexpression may be a contributor to the development of hyperplastic breast lesions. Whether its activation occurs early or late in tumorigenesis, cyclin D1 presumably acts with other oncogenic lesions to induce a fully malignant phenotype. □

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Solution structure of a pleckstrin-homology domain

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PLECKSTRIN¹, the major protein kinase C substrate of platelets, contains domains of about 100 amino acids at the amino and carboxy termini that have been found in a number of proteins, including serine/threonine kinases, GTPase-activating proteins, phospholipases and cytoskeletal proteins²⁻⁵. These conserved sequences, termed pleckstrin-homology (PH) domains, are thought to be involved in signal transduction. But the details of the function and binding partners of the PH domains have not been characterized. Here we report the solution structure of the N-terminal pleckstrin-homology domain of pleckstrin determined using heteronuclear three-dimensional nuclear magnetic resonance spectroscopy. The structure consists of an up-and-down β -barrel of seven antiparallel β -strands and a C-terminal amphiphilic α -helix that caps one end of the barrel. The overall topology of the domain is similar to that of the retinol-binding protein family of structures⁶⁻¹⁰.

The ¹H, ¹³C and ¹⁵N chemical shifts of the pleckstrin homology domain were assigned using double- and triple-resonance three-dimensional nuclear magnetic resonance (NMR) experiments¹¹⁻¹⁶ on samples of uniformly ¹⁵N- and ¹⁵N-¹³C-labelled protein. The elements of regular secondary structure were identified from the H α , C α and CO chemical shifts^{17,18}, pattern of nuclear Overhauser effects¹⁹, vicinal H^N, H α coupling constants¹⁹, and amide-exchange rates. The secondary structure consists of a seven-stranded antiparallel β -sheet and a C-terminal α -helix (Fig. 1). The observed secondary structure is similar to predictions based on residue conservation periodicity⁴.

The three-dimensional structure of the PH domain was determined using a distance geometry/simulated annealing protocol^{20,21} from a total of 1,376 NMR-derived restraints. The structures satisfy the distance restraints with no violation greater than 0.3 Å and have good covalent geometry and non-bonded

TABLE 1 Structural statistics and r.m.s. deviation for 25 PH-domain structures

Structural statistics	$\langle SA \rangle$	$\langle SA \rangle_r$
X-PLOR energies (kcal mol ⁻¹)		
E_{tot}	199 $\pm$ 8	214
E_{vdw}^*	25 $\pm$ 3	26
$E_{\text{cdih}}^\dagger$	0.2 $\pm$ 0.1	0.3
$E_{\text{noe}}^\ddagger$	14 $\pm$ 1	30.3
r.m.s. deviation from idealized values		
Bonds (Å)	0.03 $\pm$ 0.00	0.03
Angles (°)	0.47 $\pm$ 0.01	0.48
Impropers (°)	0.45 $\pm$ 0.03	0.43
Cartesian coordinate r.m.s.d. (Å)		
$\langle SA \rangle$ versus $\langle SA \rangle_\$$	0.79 $\pm$ 0.1	1.36 $\pm$ 0.1
$\langle SA \rangle$ versus $\langle SA \rangle_\parallel$	0.55 $\pm$ 0.1	1.09 $\pm$ 0.1
	N, C α , C'	Heavy atoms

Where $\langle SA \rangle$ is the ensemble of 25 final X-PLOR structures generated from the DG/SA protocol as described in the X-PLOR 3.1 manual²⁶, $\langle SA \rangle_\$$ is the cartesian coordinates obtained by averaging $\langle SA \rangle$ following a least-squares superposition of the backbone heavy atoms (N, C α , C') for residues 4–104; $\langle SA \rangle_r$ is the energy-minimized averaged cartesian coordinates.

* The X-PLOR F_{repl} function was used to simulate van der Waals interactions with atomic radii set to 0.8 times their CHARMM²⁷ values.

† Torsional restraints were applied to 45 ϕ angles with bounds of $-120 \pm 30^\circ$ for those angles with ³J_{HN,HA} coupling constants >9.0 Hz, and $-60 \pm 30^\circ$ for coupling constants <5.5 Hz. Restraints for the latter were applied only in the α -helical region. Force constants of 200 kcal mol⁻¹ rad⁻² were used for all torsional restraints.

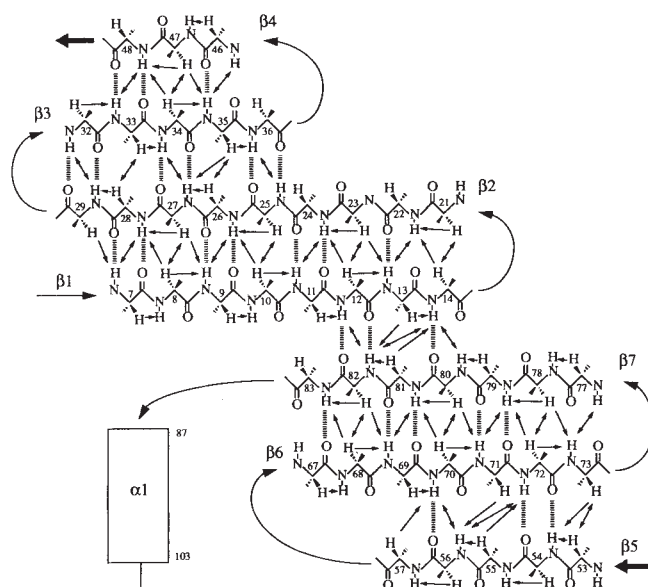
‡ A total of 1,255 NOE-derived distance restraints were applied with a square well potential and a force constant of 50 kcal mol⁻¹ Å⁻². Of these restraints, 361 were intra-residue, 317 were sequential, 159 were between residues separated by less than 5 residues in the primary sequence, and 418 were from long range NOEs. Lower bounds for all NOE-derived restraints were set to 1.8 Å. Distance restraints derived from the ¹⁵N resolved 3D-NOESY and the 60 ms ¹³C-resolved 3D-NOESY were given upper bounds of 2.5, 3.3, 4.0 or 5.0 Å depending on NOE crosspeak intensity. Distance restraints derived from the 80 ms ¹³C-resolved NOESY were given upper bounds of 5.0 Å regardless of crosspeak intensity. Corrections for centre averaging²⁸ were applied where appropriate. 38 hydrogen bonds were included and given bounds of 1.8–2.3 (H $\rightarrow$ O) and 2.7–3.3 (N $\rightarrow$ O) Å with force constants of 50 kcal mol⁻¹ Å⁻². No distance restraint was violated by >0.3 Å in any of the final structures.

§ R.m.s.d. for residues 4–104.

|| R.m.s.d. for residues 4–14, 21–58 and 67–104.

FIG. 1 Secondary structure of the PH domain. NOEs that define the structure of the β -sheet are indicated by arrows. The characteristic NOEs expected for an α -helix (d_{NN} , $d_{\text{NN}(i+3)}$, $d_{\text{NN}(i+4)}$)²¹ were observed for residues 87–103. Hydrogen bonds supported by the amide exchange data that were included in the structure calculations are indicated by dashed vertical lines.

METHODS. All NMR spectra were acquired at 30 °C on a Bruker AMX 500 or AMX600 NMR spectrometer. The ¹H, ¹³C and ¹⁵N resonances of the backbone were assigned by correlating the amide ¹H and ¹⁵N resonances of each amino acid to the C α , H α and C' signals of the i and $(i-1)$ residues from a series of 3D NMR experiments (NOESY-HSQC, TOCSY-HSQC, HNCA, HN(CO)CA, HNCO, HN(CA)CO, HCACO)¹¹⁻¹⁴. The side-chain signals were assigned from 3D HCCH-TOCSY, CBCA(CO)NH, CCH-COSY and HNHB experiments^{15,16}. Uniformly ¹⁵N- and ¹⁵N-¹³C-labelled proteins were prepared for the NMR experiments by growing bacteria that overexpress the PH domain in a minimal medium containing ¹⁵NH₄Cl with or without [U-¹³C] glucose. The PH domain was cloned from poly(A)⁺ RNA of HL60 promyelocytic leukaemia cell line using reverse transcriptase-PCR. The cDNA of the PH domain corresponding to residues 1–105 was subcloned into pE20b plasmid and expressed in *Escherichia coli* HMS174(DE3) cells with an additional Leu-Glu-(His)₆ sequence at the C terminus. The PH domain was purified by affinity chromatography on a nickel-IDA column (Invitrogen) and exchanged into phosphate buffer (20 mM, pH = 6.5) containing 100 mM NaCl and 5 mM perdeuterated DTT.



contacts (Table 1). Figure 2*a* and *b* shows two stereoviews of the backbone superposition of the 25 lowest energy structures of the PH domain. The structure of the backbone is well defined by the NMR data except for the first three residues at the N terminus and the last nine residues at the C terminus, which

includes the six-histidine tag. The side chains for the internal hydrophobic residues are also well defined by the NMR data (Fig. 2*c*). The atomic root-mean-square (r.m.s.) distribution about the mean coordinate positions for residues 4–104 of the PH domain is 0.79 ± 0.1 Å for the backbone atoms and

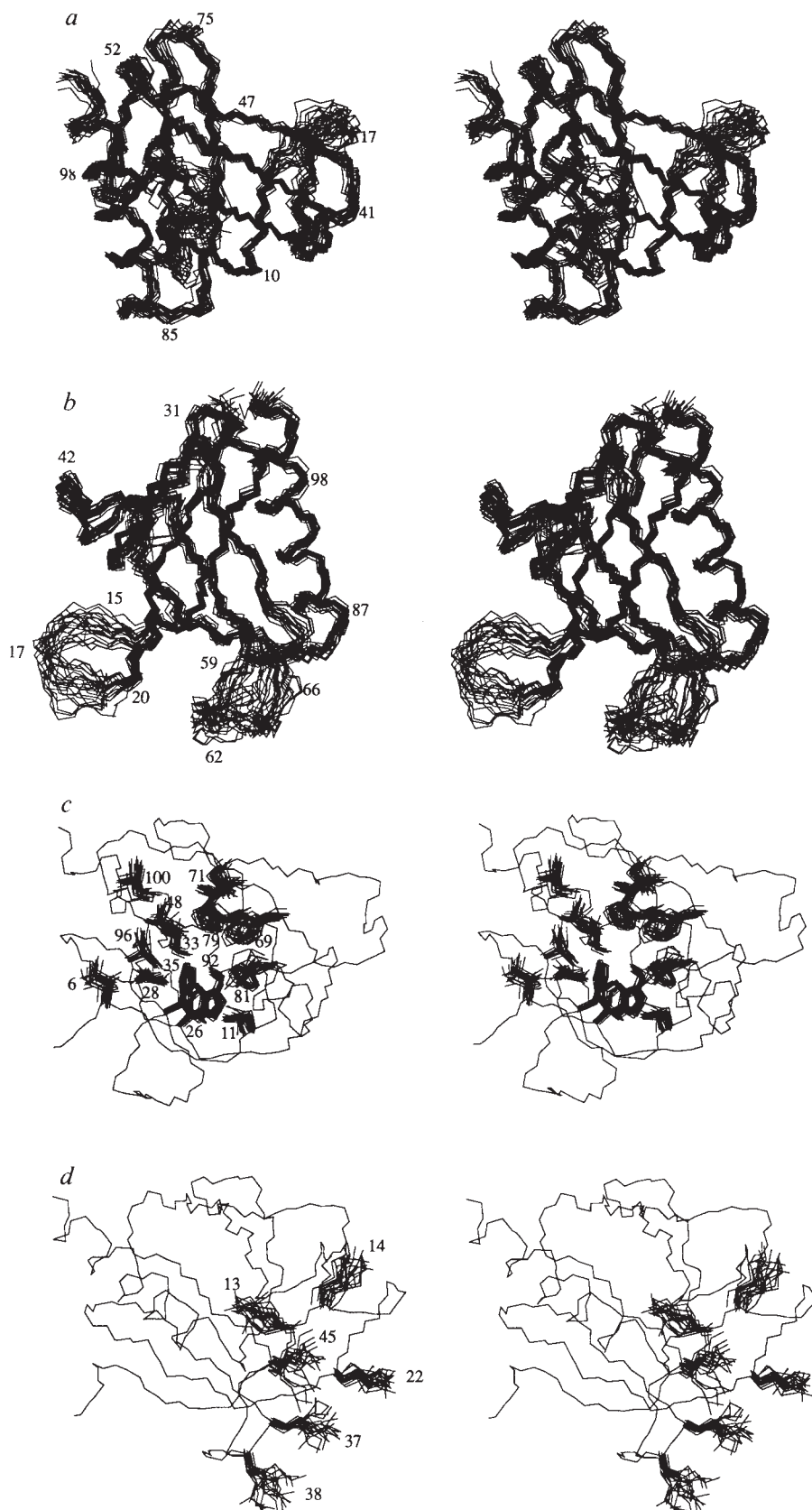


FIG. 2 Stereoviews of the backbone (N, C α , C') (a, b) and selected sidechains (c, d) of 25 superimposed NMR-derived structures (residues 4–104) of the N-terminal PH domain of pleckstrin. These structures were generated with a distance geometry/simulated annealing (DG/SA) protocol^{20,21} using the X-PLOR program²⁶. The structure calculations used 1,255 proton–proton distance restraints obtained from ¹⁵N- and ¹³C-resolved heteronuclear three-dimensional nuclear Overhauser effect (NOE) spectra, 38 hydrogen bonds from an analysis of the amide exchange rates measured from a series of ¹H/¹⁵N HSQC spectra recorded after the addition of ²H₂O, and 45 ϕ angle restraints from the ³J_{H_N,H α} coupling constants measured in an HMQC-J experiment²⁹. The coordinates have been deposited in the Brookhaven Protein Databank, accession number 1PLS.

1.36 ± 0.1 Å for all heavy atoms. If two loops (residues 15–20 and 59–66) are excluded, the r.m.s. distribution about the mean drops to 0.55 ± 0.1 Å for the backbone and 1.09 ± 0.1 Å for all heavy atoms.

The N-terminal PH domain of pleckstrin forms an up-and-down β -barrel composed of two orthogonal β -sheets (Fig. 3a). In the orientation shown in Fig. 3a, the front β -sheet is composed of the first four strands, and the back sheet is defined by the last three strands together with part of the first and second strand. At the C terminus of the protein, an amphiphilic α -helix (residues 87–103) caps one end of the barrel with the hydrophobic side chains of Trp 92, Ile 96 and Ile 100 pointing into the core of the barrel. Hydrophobic residues are located in the interior of the β -barrel as well as in a cluster (Val 12, Lys 14, Trp 21, Phe 62, Lys 70, Phe 80) on the outside of the barrel. A *cis* peptide bond is observed between Ser 57 and Pro 58. However, this is unlikely to be of any functional significance because a Pro residue at this position is not conserved in other PH domains^{2–5}. In fact, the only amino acid that is invariant in all of the PH domains is Trp 92 of the C-terminal α -helix. The side chain of this residue interacts with the hydrophobic core of the β -barrel and, like many of the other conserved hydrophobic residues that form the core of the barrel (Figs 2c and 3b), most probably contributes to protein stability. Another interesting feature of the structure is the presence of six lysine residues near the entry of the β -barrel (Fig. 2d). Four of these lysines (13, 14, 22 and 45) are conserved in many of the PH domains^{2–5}. The least conserved regions are found in the loops connecting the β -strands. These loops are the regions where sequence alignment indicates insertions and deletions in the different PH domains^{2–5}. However, it is expected that these differences in amino-acid sequence could be tolerated in the structure without disrupting the overall fold, suggesting that the PH domains may all possess similar three-dimensional structures.

As shown in Fig. 3, the up-and-down β -barrel of the PH domain resembles that found in retinol-binding protein (RBP)⁶.

Compared to RBP, the PH domain contains one fewer β -strand, and all strands in the PH domain are shorter in length. Also, the location of the C-terminal α -helix is different. In the PH domain, the α -helix caps one end of the β -barrel (Fig. 3a, b); whereas, the α -helix of RBP packs parallel to the β -strands on the outside of the barrel (Fig. 3c, d). However, in spite of these differences, the overall topology and dimensions of the β -barrel core are similar. Note that members of this family of proteins, which include RBP⁶, lactoglobulin⁷, bilin binding protein⁸, P2 myelin protein⁹, and fatty acid binding protein¹⁰, all bind lipophilic molecules. The binding site for retinol is located in the hydrophobic core of the β -barrel which is ideally suited for binding small lipophilic molecules (Fig. 3c, d). On the basis of the structural similarity between the PH domains and the RBP family, it may be possible that the PH domains bind to lipid molecules in the hydrophobic core of the β -barrel in an analogous fashion to members of this family. The lysine residues located at the lip of the β -barrel may also contribute to lipid binding through interactions with polar headgroups. Indeed, several proteins with PH domains (for example phospholipases and the β -adrenergic receptor kinase) are regulated by G-protein $\beta\gamma$ subunits or participate in the function of p21^{ras} (such as GTPase-activating proteins and guanine-nucleotide-releasing factors), all of which have attached lipophilic moieties. Moreover, many of the proteins that contain PH domains are localized at the membrane^{2–5,22}. This localization could occur through direct binding to lipids or to proteins already associated with the membrane. Recent experimental evidence on the β -adrenergic receptor kinase (β ARK)²³ and other PH domains²⁴ has suggested that the C-terminal helix mediates this association through protein-protein interactions with $\beta\gamma$ subunits. In these studies only the C-terminal portion of the PH domains and residues beyond the C-terminus were necessary for binding to $\beta\gamma$ subunits. Thus, the role of the N terminus, which appears to be important on the basis of mutagenesis of Btk²⁵, was not addressed. Clearly more work is needed to identify the binding partners and

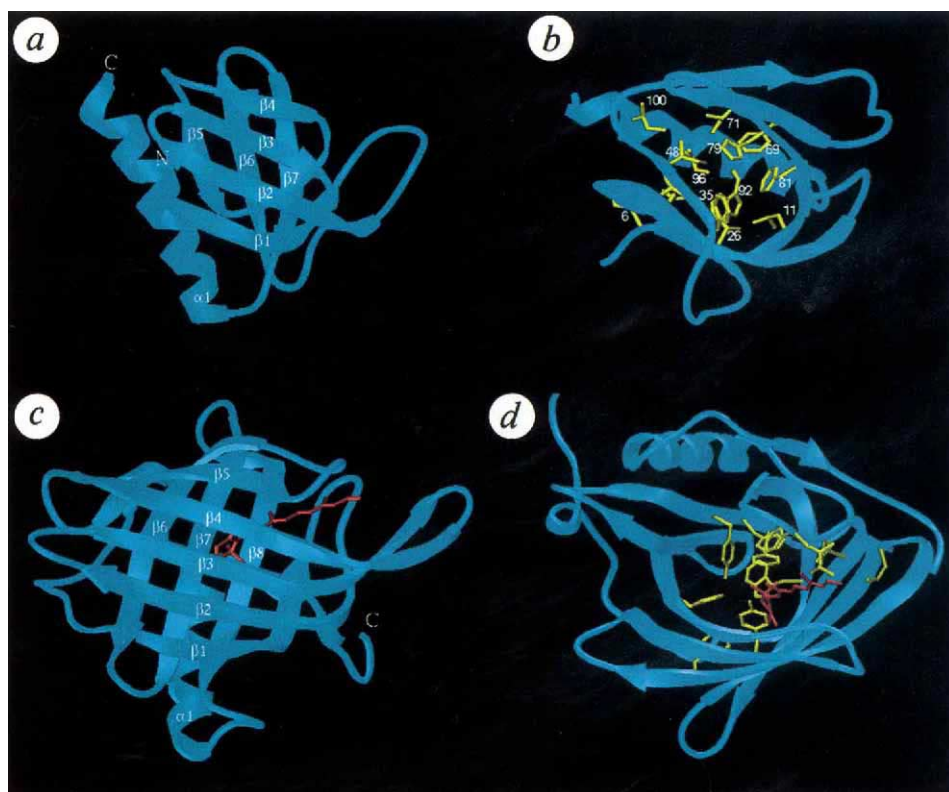


FIG. 3 Ribbon plots³⁰ depicting the averaged minimized NMR structure of the pleckstrin homology domain (residues 4–104) (a, b) and the X-ray structure of the retinol-binding protein complexed to retinol (red) (c, d)⁶. β -strands 1–4 and 5–7 of the PH domain (a) correspond to strands 1–4 and 6–8 of the retinol-binding protein (c). Side-chain atoms are shown in yellow for selected hydrophobic residues located in the core of the β -barrel.

portions of the PH domains that are responsible for binding to other molecules. Site-directed mutagenesis studies, guided by the NMR-derived structure reported here, should be helpful in characterizing the important residues that mediate binding and biological activity in this interesting class of protein modules. □

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Structure of the pleckstrin homology domain from β -spectrin

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THE 'pleckstrin homology' or PH domain is a 100-residue protein module. It is present in many kinases, different isoforms of phospholipase C, GTPase-activating proteins and nucleotide-exchange factors^{1–4}. Its function is not known, but many proteins that contain a PH domain interact with GTP-binding proteins⁵. The PH domain in β -adrenergic receptor kinase may be involved in binding to the β subunits of a trimeric G-protein^{3,4,6,7}. We report here the three-dimensional structure of the PH domain of the cytoskeletal protein spectrin using homonuclear nuclear magnetic resonance. The core of the molecule is an antiparallel β -sheet consisting of seven strands. The C terminus is folded into a long α -helix, and another helix is present in one of the surface loops. The molecule is electrostatically polarized and contains a pocket which may be involved in the binding of a ligand. There is a distant relationship to the peptidyl-prolyl-*cis-trans*-isomerase FKBP in which this pocket is involved in the binding of the macrocyclic compound FK506 (refs 8–11).

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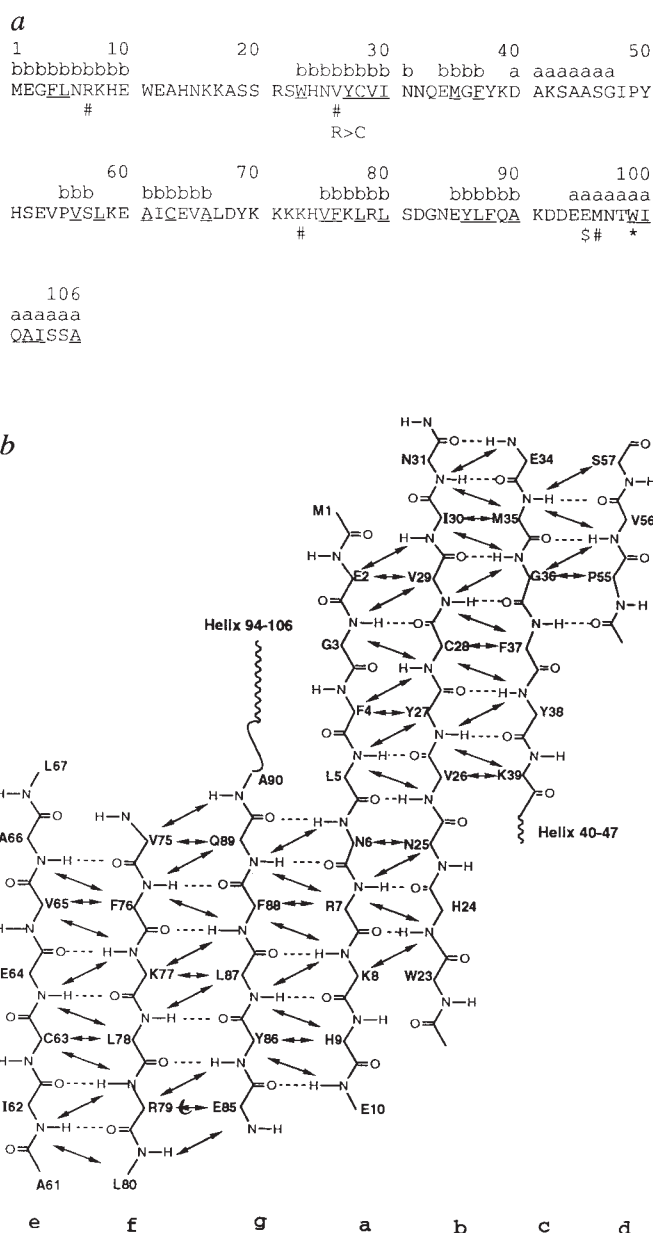
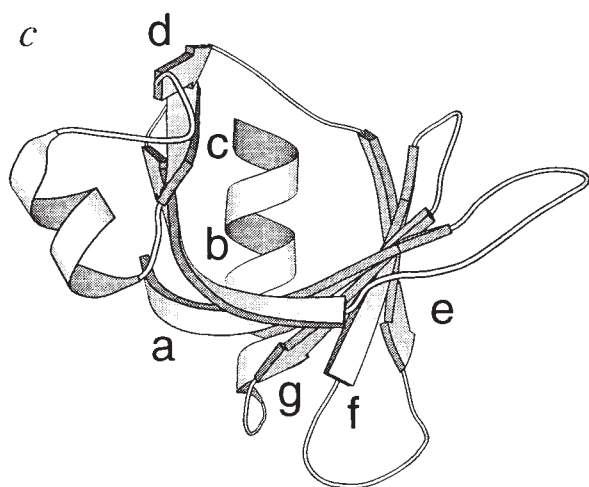
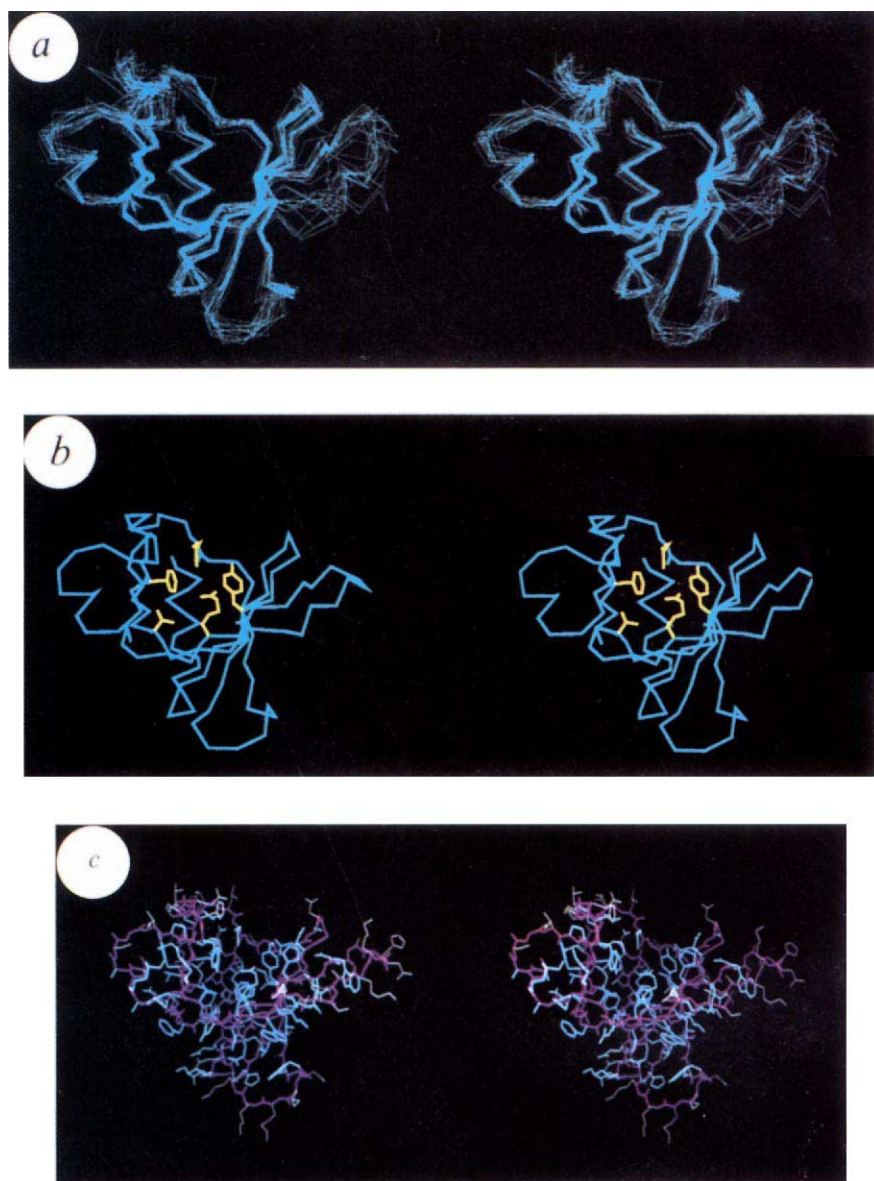


FIG. 1 **a**, Amino-acid sequence of the mouse brain spectrin PH domain. The underlined positions have conserved hydrophobic residues in the multiple alignment³. The positions of conserved basic residues are labelled with #, the conserved acidic position with \$ and the invariant tryptophan with *. The position of the *xid* mutation (R>C) is shown. Residue numbering and secondary structure are shown above the sequence (a indicates α -helix and b β -strand). **b**, Secondary structure of the domain. The hydrogen bridges indicated in the picture are derived by the slow proton exchange rates (>weeks) and interstrand NOEs. **c**, A MOLSCRIPT (ref. 24) plot of the structure. The strands of the β -sheet are labelled a–g according to their occurrence in the sequence.

METHODS. The nucleotide sequence coding for the PH domain was amplified with the polymerase chain reaction using mouse brain cDNA as a template. This sequence was inserted in the pET21d vector (Novagen, Madison, WI) and expressed in *E. coli* BL21 (DE3) essentially as described before²³. The PH domain was found in the soluble extract after the cells had been broken with a French press. It was precipitated with ammonium sulphate at 50–80% saturation and dissolved into a buffer containing 0.1 M diethanolamine (pH 9.0), 1 mM dithiothreitol (DTT) and 1 mM EDTA plus the proteolytic inhibitors phenylmethyl sulphonyl fluoride (0.1 mM) and benzamidin (0.1%). Further purification was done in this buffer. Three column steps were necessary for purification: first, anion-exchange on Q-Sepharose HP, followed by gel filtration on Superdex 75 and anion-exchange on Mono-Q (details of the ►

FIG. 2 a, Superposition of 14 structures selected on the basis of minimal total energy (stereo view). The large insertions, which are specific to the spectrin domain³, are at the upper left edge (a small helix followed by a loop) and in the rightmost strand, which shows structural fluctuation. The structures were calculated using 605 inter-residue NOEs in the first calculation step, and refined with 1,252 NOEs, using the program X-PLOR (ref. 25) essentially as described before²⁶. In the additional refinement step, ambiguous crosspeaks were treated as described²⁷. The average r.m.s. differences from the mean structure are 0.69 Å (± 0.23) for backbone atoms, and 1.00 Å (± 0.33) for all non-hydrogen atoms in the well defined core of the module, and 0.99 Å (± 0.34) and 1.58 Å (± 0.33), respectively, for all residues. The core consists essentially of the β -sheet region and the C-terminal α -helix (residues 2–12, 22–45, 48–50, 55–67, 72–106). b, A selected structure closest to the average structure. It includes the side chains V56, Y86, R7, V26 and F37 (clockwise; single-letter amino-acid code). The positions of these side chains correspond to the residues in FKBP that are involved in the ligand binding. The orientation of the domain is the same as in a. c, The same structure as in b, but with all side chains added. The side chains are colour-coded according to the r.m.s. deviations of all non-hydrogen atoms in the set of calculated structures from the average structure: dark blue <0.75 Å; light blue <1.5 Å, grey >1.5 Å.



purification protocol are available from the authors). For the NMR experiments, the protein was changed into a buffer containing 10 mM potassium phosphate (pH 6.5) and 1 mM DTT using gel filtration.

The PH domain is close to the carboxy terminus of the β -subunit of the non-erythrocyte spectrin (fodrin). The domain of the mouse brain spectrin was expressed in *Escherichia coli* and purified as described in the legend to Fig. 1. Our construct contains 106 amino-acid residues (Fig. 1a). Similar to the two domains in pleckstrin³, the spectrin domain has two insertions of about eight residues (positions 12–20 and 41–48). The sequence is rich in aromatic residues distributed over a wide range of the spectrum (5.5–8 p.p.m.), which causes the proton nuclear magnetic resonance (NMR) spectrum to be well dispersed. This and the fact that the amide nitrogens in the β -sheet (Fig. 1b) do not exchange their protons in D₂O greatly helped the spectral assignment. Thus, we were able to assign the spectrum using two-dimensional COSY, TOCSY and NOESY techniques supported by three-dimensional TOCSY–NOESY (refs 12–15). The side-chain assignments are complete except for those of residues 21, 33 and 72. The structure calculations lead to a well defined structure of the basic structural frame as shown in Fig. 2a. Two loop regions (a–b and c–d; Fig. 1c) with insertions specific for the spectrin domain are less well defined.

The basic structural features of the PH domain are shown in Fig. 1c. The core of the molecule is made of a seven-stranded antiparallel β -sheet. A hydrophobic core is formed by residues Leu 5, Met 35, Tyr 37, Phe 76, Leu 78, Tyr 86, Phe 88, Try 99,

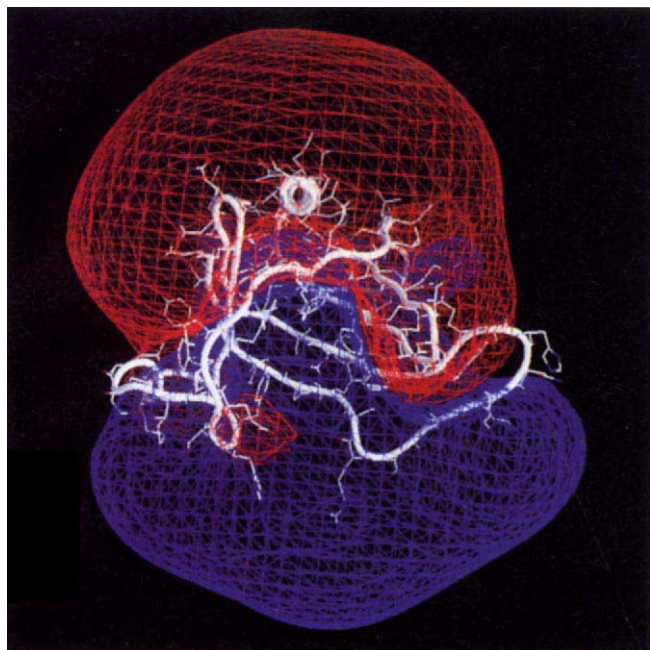


FIG. 3 Electrostatic potential contoured and colour-coded at -1.5 kT (red) and $+1.5$ kT (purple). The potential was calculated and displayed with the program GRASP^{28,29}. Compared with Fig. 2, the molecule has been turned about 90° so that the view is to the putative binding pocket from the top of the molecule.

Ile 100 and Ile 103. Their interactions cause a strong bending of the sheet into a half-moon shape. The strands are labelled a–g in the order they appear in the sequence. Their order is e–f–g–a–b–c–d in the sheet. The hydrogen bonding and NOE pattern show a very regular antiparallel β -sheet (Fig. 1b).

The C-terminal residues form a long α -helix which is probably highly conserved among the homologous PH domains³. It is packed into a gorge below the plane of the strands a and b in parallel to strand e. The spectrin PH domain also contains a well defined two-turn α -helix in the loop between strands c and d. This loop is much shorter in most of the other PH domains, suggesting that the helix is not present in them. Multiple alignments of PH domain sequences^{1–4} (about 60 PH domains have currently been identified in diverse proteins; T. Gibson, personal communication) indicate that all loops except the tight bend between strand g and the C-terminal helix tolerate insertions. The loop between strands c and d in the PH domain of phospholipase C γ has a very long insertion which contains two Src-homology 2 (SH2) domains and one SH3 domain. This splits the PH domain of PLC γ into two halves.

The revised alignment predicts that the first β -strand is two or three residues longer than the one expressed here, and that the C-terminal α -helix also has a further turn. These extensions still place the N and C termini close to each other, a feature common to other domains (such as SH2 and SH3) in different host proteins.

The function of the PH domain is not known. However, some recent results are relevant to the discussion on its function. First, a PH domain has been found^{3,4} in the C-terminal region of β -adrenergic receptor kinase. Exactly this region is involved in the attachment of this kinase to the membrane-bound $\beta\gamma$ subunits of a heterotrimeric G-protein^{6,7}. Further, although much more circumstantial, evidence for the interaction of the PH domain and the $\beta\gamma$ complex derives from the activation of the enzymes belonging to the β subclass of phospholipase C. They are activated by direct interaction with the complex^{16,17} and may contain an N-terminal PH domain¹⁸. The second point is that the C-terminal part of the β -subunit of brain spectrin (which contains the PH domain) has been shown to bind to alkali-washed brain membranes¹⁹. Whether this is due to the polarized nature of the protein (Fig. 3) or whether the PH domain binds to a membrane receptor, is not known.

The third piece of information comes from the localization of the *xid* mutation in Bruton's agammaglobulinaemia tyrosine kinase (Btk) that leads to perturbation of B-cell maturation and immune deficiency^{20,21}. This point mutation takes place within a PH domain³ and substitutes an arginine residue with a cysteine at a conserved Arg/Lys position of the domain (position 26 in Fig. 1a; valine in the spectrin PH). The residue at position 26 is fixed in the middle of strand b and exposed to the surface. Therefore, it is unlikely that an Arg→Cys substitution would have a merely structural consequence. The *xid* mutation indicates that the PH domain interacts with a specific ligand such as another protein.

The structural analysis gives further evidence for the interaction of the PH domain with a specific ligand. A search for similar structures in the PDB data base using the computer program DALI (ref. 22) revealed a distant relationship to the FK506-binding protein (FKBP) which has a similar shape but different topology^{10,11}. The immunosuppressant binds to a similar pocket in FKBP. The structural alignment shows that the residues involved in the binding of FK506 in FKBP (Tyr 26, Phe 46, Val 55, Ile 56, Try 59 (refs 8–11)) correspond to the residues Arg 7, Val 26, Phe 37 (a replacement for Val 55, Ile 56 and Try 59), Val 56, Lys 59 (a replacement for Try 59) and Tyr 86 in the PH domain (Fig. 2b). Val 26 is the site of the *xid* mutation.

The PH structure can be seen as a large binding pocket assembled by a relatively small, rigid framework (Figs 1c, 2c). Figure 3 shows the strong electrostatic polarization of the molecule; the positively charged (purple) and negatively charged (red) ends of the domain surround the pocket. We speculate that this pocket is involved in binding a specific ligand such as a peptide. □

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A serine kinase regulates intracellular localization of splicing factors in the cell cycle

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SMALL nuclear ribonucleoprotein particles (snRNPs) and non-snRNP splicing factors containing a serine/arginine-rich domain (SR proteins) concentrate in 'speckles' in the nucleus of interphase cells¹. It is believed that nuclear speckles act as storage sites for splicing factors while splicing occurs on nascent transcripts². Splicing factors redistribute in response to transcription inhibition^{3,4} or viral infection⁵, and nuclear speckles break down and reform as cells progress through mitosis⁶. We have now identified and cloned a kinase, SRPK1, which is regulated by the cell cycle and is specific for SR proteins; this kinase is related to a *Caenorhabditis elegans* kinase and to the fission yeast kinase Dsk1 (ref. 7). SRPK1 specifically induces the disassembly of nuclear speckles, and a high level of SRPK1 inhibits splicing *in vitro*. Our results indicate that SRPK1 may have a central role in the regulatory network for splicing, controlling the intranuclear distribution of splicing factors in interphase cells, and the reorganization of nuclear speckles during mitosis.

SR splicing factors are phosphoproteins containing consensus sequences (S/T*-P-X-R/K (asterisk indicates the phosphorylation site)) for the important mitotic kinase p34^{cdc2} (ref. 8). Surprisingly, p34^{cdc2} activity in extracts from metaphase HeLa cells, assayed by its ability to phosphorylate histone H1, could not phosphorylate SC35, a member of the SR family of proteins (Fig. 1A). In addition, depletion of p34^{cdc2} from these extracts had no effect on their ability to induce disassembly of nuclear speckles in Triton X-100-permeabilized cells grown on coverslips (data not shown), suggesting that p34^{cdc2} may not be directly involved in the phosphorylation of SR proteins or in the disassembly of nuclear speckles.

Fractionated mitotic extracts revealed a kinase activity for SC35 separated from the p34^{cdc2} activity (Fig. 1A). The p34^{cdc2}-containing fractions could induce disassembly of nuclear lamina (Fig. 1Ba), consistent with previous reports^{9,10}, but had no effect on nuclear speckles (Fig. 1Bb). In contrast, the SC35-kinase-containing fractions induced disassembly of nuclear speckles but not nuclear lamina (Fig. 1Bd and e). These results indicated that nuclear structures containing splicing factors may be controlled by an enzyme distinct from p34^{cdc2}, and that the speckle disassembly activity cofractionated with a major kinase activity for SC35.

To determine the specificity of this kinase, we examined a number of substrates (Fig. 1C). Phosphorylation of the retinoblastoma gene product (Rb) is cell-cycle-dependent and regulates its association with nuclear structures¹¹ and the protooncogene product c-Jun is phosphorylated during serum stimulation¹²; however, neither protein was phosphorylated by the isolated SC35 kinase (Fig. 1C). As the kinase phosphorylates members of the SR family of splicing factors¹³ and U2AF65, a non-snRNP splicing factor with a serine- and arginine-rich

domain¹⁴ (Fig. 1C), we designated it SR-protein-specific kinase, SRPK1.

Phosphoamino-acid analysis of SC35 (Fig. 1D) and other SR proteins (data not shown) phosphorylated by SRPK1 *in vitro* showed that phosphorylation took place exclusively on serine residues. Removal of the serine and arginine repeats from the SR protein SF2/ASF resulted in a mutant protein which could no longer be phosphorylated by SRPK1 (Fig. 1E), suggesting that SRPK1 recognizes the conserved SR domain. A U1-associated kinase activity has been reported that specifically recognizes this domain in SF2/ASF¹⁵. As SRPK1 was the only major kinase for SR proteins found during purification (Fig. 1A), it may be responsible for this U1-associated kinase activity.

SRPK1 activity was also present in whole cell and nuclear extracts from unsynchronized HeLa cells (data not shown), suggesting that it functions in interphase cells. As the SR domain is responsible for targeting SR proteins to nuclear speckles¹⁶ and for protein-protein interactions with splicing factors containing a similar SR domain¹⁷⁻¹⁹, SRPK1 may in turn be involved in the regulation of these interactions and spliceosome assembly events.

We purified SRPK1 to homogeneity by a series of chromatography steps (manuscript in preparation); silver staining of the purified kinase revealed a single band of relative molecular mass 92,000 (*M_r* 92K) (Fig. 1f). A 4.3-kilobase (kb) complementary DNA with an open reading frame encoding a protein of 655 amino acids (Fig. 2a) was isolated by screening human cDNA libraries with an oligonucleotide probe based on a unique peptide sequence from the purified protein. This cDNA appears to be full-length as a 4.5-kb poly(A)⁺ messenger RNA was detected by northern blotting (data not shown). The calculated *M_r* of the encoded protein is 74.3K, although the *in vitro* translated protein migrated similarly to purified SRPK1 on an SDS-polyacrylamide gel (data not shown). The deduced protein sequence contains all three peptide sequences and conserved residues of the catalytic domains of serine/threonine kinases²⁰. In addition, two stretches of basic amino-acid sequences may function as nuclear targeting signals (Fig. 2a).

A search of sequence databases revealed that SRPK1 is a new protein which is highly related to a hypothetical *C. elegans* kinase (CEHK) and to the fission yeast kinase Dsk1 (ref. 7) (Fig. 2b). The overall similarity between these three kinases suggests that they may be homologues. Interestingly, Dsk1 was isolated as a multicopy suppressor for a cold-sensitive *dis1* mutant that affects chromosome segregation during mitosis⁷ and, although *dsk1*⁺ gene is not essential for viability, overexpression of *dsk1* causes a delay in progression through the G2/M phases of the cell cycle. Dsk1 was mostly found in the cytoplasm of interphase cells but was localized in the nucleus of mitotically arrested cells. When the spacer sequence between the kinase domains VIb and VII (Fig. 2b) was deleted, mutant Dsk1 was enriched in the nucleus; similar experiments remain to be done on SRPK1.

Cells arrested at interphase (I) or metaphase (M) were labelled metabolically with [³²P]orthophosphate and SR proteins were isolated by a two-step precipitation with salt¹⁴. The results in Fig. 3a show that SR proteins were hyperphosphorylated 3-5-fold in metaphase as compared to interphase cells, and that purified SR proteins were phosphorylated on serine residues (data not shown). Phosphorylation of SR proteins *in vivo* is therefore cell-cycle regulated.

SRPK1 activity, assayed using SC35 or SF2/ASF as substrates was also activated 3-5-fold in extracts from metaphase compared to interphase cells (Fig. 3b). Dsk1 was similarly regulated during the cell cycle⁷, consistent with SRPK1 and Dsk1 being functional homologues. The association of phosphorylation of SR proteins *in vivo* with SRPK1 activity in the extracts, together with the fact that SRPK1 was the only major kinase for SR proteins obtained during purification (Fig. 1a), strongly suggests that SRPK1 is responsible for phosphorylation of SR proteins during the cell cycle *in vivo*.

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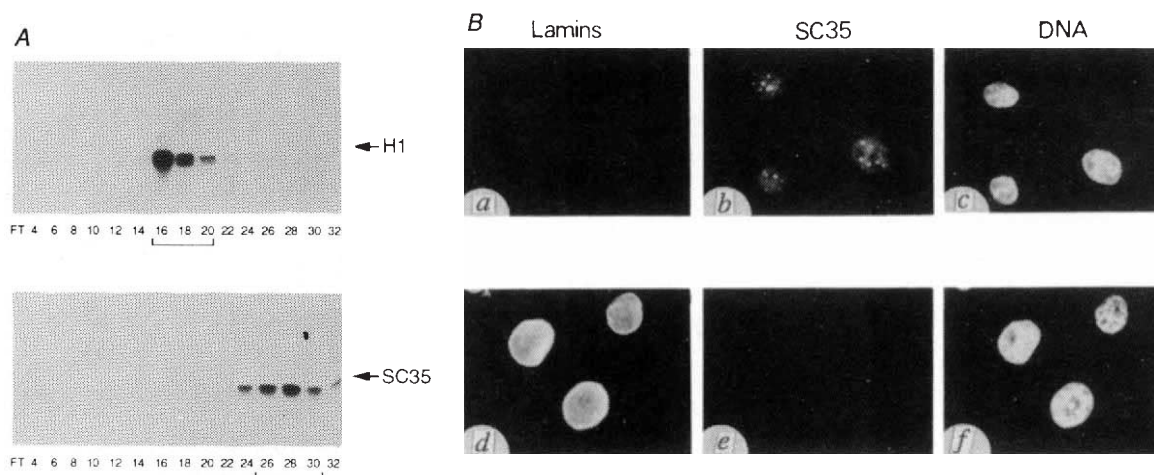
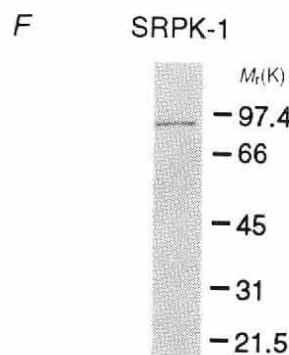
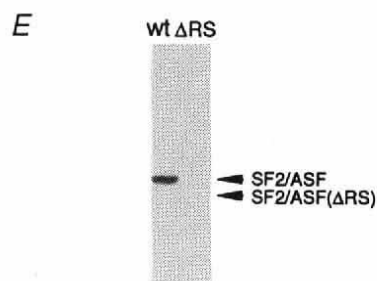
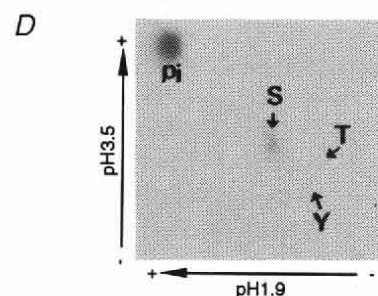
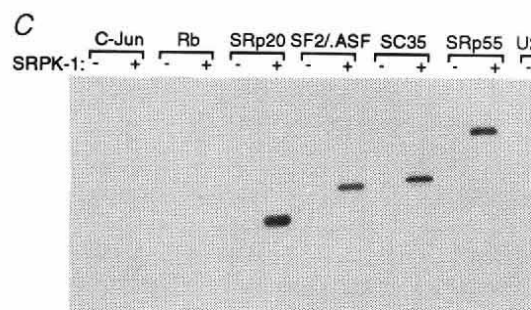


FIG. 1 Identification and characterization of the new kinase. **A**, Fractionation of mitotic extracts on a phosphocellulose P11 column: H1 kinase elutes at 0.35 M NaCl (top); SC35 kinase elutes at 0.6 M NaCl (bottom). **B**, MG63 cells were triple-stained for nuclear lamina (a, d) using human anti-lamin A/C sera, for SC35 (b, e) using anti-SC35 monoclonal antibody, and for DNA (c, f) with Hoechst 33258. Human and mouse antibodies were probed with anti-human immunoglobulin-fluorescein and anti-mouse immunoglobulin-rhodamine, respectively. Partially purified H1 kinase (fractions 16–20) induced disassembly of nuclear lamina (a) but had no effect on nuclear speckles (b); SC35 kinase (fractions 26–30) had no effect on nuclear lamina (d) but induced disassembly of nuclear speckles (e). DNA condensation was not evident by either treatment (c, f). **C**, Substrate specificity of SRPK1. c-Jun and Rb recombinant proteins were expressed and purified from bacteria. SR proteins and U2AF65 were recombinant proteins purified from baculovirus-infected Sf9 cells. **D**, Phosphoamino-acid analysis of SC35 phosphorylated *in vitro* by SRPK1. Released phosphate (P_i), phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated. Labelled phosphoserine was detected, indicating that SC35 was phosphorylated exclusively on serine residues. **E**, Wild-type, but not mutant Δ RS SF2/ASF in which the serine and arginine repeats in the SR domain were deleted²⁶, was phosphorylated *in vitro* by SRPK1. **F**, Peak fraction of SC35 kinase from the final Mono-S purification step was silver stained to reveal a 92K band.

METHODS. Cell extracts were prepared from HeLa cells grown in 150-mm culture dishes to 70% confluency. Cells were arrested at M phase by adding nocodazole (600 ng ml⁻¹) to fresh medium and continuing to culture for 12 h after thymidine block²⁷. Cell extracts were prepared from metaphase cells as described²⁸. Mitotic extracts (4 ml) were loaded onto a 5-ml phosphocellulose P11 (Waterman) column equilibrated in buffer B (20 mM Tris-HCl, pH 7.2, 5 mM β -glycerophosphate, 1 mM EGTA, 1 mM DTT, 1.5 mM MgCl₂) plus 50 mM NaCl. After washing, the column was developed with a gradient of NaCl from 50 mM to 1 M. Aliquots were taken from alternate 1.5-ml fractions for kinase assay using histone H1 (Boehringer) or recombinant SC35 prepared from a baculovirus expression system²² and purified on a C4 HPLC column as substrates. Kinase activity was assayed at room temperature for 15 min in 20 μ l containing 2 μ l column fraction, 0.5 μ g H1 or SC35, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM DTT, 2 μ M ATP, 2 μ l [³²P- γ]ATP, stopped with SDS loading buffer, the proteins separated on 12.5% SDS-polyacrylamide gels, and kinase activity detected by autoradiography. For the disassembly assay, column fractions were pooled and concentrated 5-fold in a Centricon-30 (Amicon). Proteins were desalted and the buffer changed to MEB (50 mM Tris-HCl, pH 7.3, 50 mM KCl, 10 mM MgCl₂, 20 mM β -glycerophosphate, 10 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 10 μ g ml⁻¹ aprotinin) in the same Centricon. MG63 cells were chosen for immunofluorescent microscopy because of their large and flat nuclei and were grown at 30% confluency on slides for 3 to 5 h. Cells were permeabilized and disassembly assayed according to ref. 29. Permeabilized cells were treated with 20 μ l of column fractions at 32 °C for 1 h in the presence of ATP and an ATP-regenerating system²⁸, then processed for immunofluorescence microscopy as described¹. DNA was stained by immersing slides for 1 min in 10 μ g ml⁻¹ Hoechst 33258 before mounting. To determine substrate specificity, SR proteins including SRp20, SC35, SF2/ASF and SRp55 were prepared as described²². U2AF65 coding sequence was cloned in-frame into a pBacHisB vector (Invitrogen) and the protein purified from baculovirus-infected Sf9 cells on a Ni-bead column (Invitrogen). SR and U2AF proteins were further purified on a C4 HPLC column and 0.5 μ g used for kinase assay. Kinased SC35 isolated from SDS gel slices was used for phosphoamino-acid analysis³⁰.



◀ FIG. 2 a, Cloning of SRPK1. Nucleotide (positions on the left) and deduced amino-acid (positions on the right) sequence of SRPK1 have been deposited in GenBank (accession number U09564). The sequence preceding the first ATG contains stop codons in all three reading frames. The sequence surrounding the start codon is identical to the Kozak consensus. Three peptide sequences derived from purified SRPK1 are indicated by a dashed line. Residues conserved in the catalytic domains of serine/threonine kinases are encircled. Potential nuclear localization signals are indicated by a solid line; the polyadenylation signal is underlined. b, Comparison of amino-acid sequences of *C. elegans* hypothetical kinase (CEHK; database accession number S28282), SRPK1 and fission yeast kinase Dsk1 (accession number D13447). Identical amino acids (42% with CEHK and 30% with Dsk1) are in bold. Conserved kinase domains (–XI; solid line) and residues (marked with an asterisk) are indicated²⁰. Not shown is a *C. elegans* sequence of 440 amino acids near the N terminus. Stretches of basic amino acids that

may function as nuclear localization signals are boxed.

METHODS. Purified SRPK1 was blotted onto PVDF membrane and digested *in situ* with trypsin. The resulting peptide mixture was resolved by reverse-phase HPLC and the best fractions sequenced. ³²P end-labelled 38-mer oligonucleotides (5'-ACIGCIGGIAA(T/C)TT(T/C)(T/C)TIG-TIAA(T/C)CCI(T/C)TIGA(A/G)CCIAA-3', where I is inosine) based on the unique peptide sequence STAGNFLVNPLEPK from purified SRPK1 was used to screen a Uni-ZAP XR HeLa S3 library (Stratagene). 1 × 10⁶ plaques were screened by hybridization of nitrocellulose filters at 37 °C for 48 h in 5 × SSC, 5 × Denhardt's, 50 mM NaHPO₄, pH 6.8, 0.1% SDS, 200 µg ml⁻¹ single-stranded DNA, and washed at 50 °C in 5 × SSC, 0.1% SDS to yield >30 positive clones, most of which were independent and one of which was full-length. The sense strand was sequenced from a series of 5' deletions generated by the Erase-a-Base kit (Promega) and confirmed by sequencing of the complementary strand using custom oligonucleotides.

In addition, SRPK1 purified to homogeneity was sufficient to induce disassembly of nuclear speckles (Fig. 4Aa and c), but had no effect on nuclear lamins (Fig. 4Ab and d), nuclear pore complexes, or DNA replication foci (data not shown). To determine whether splicing factors other than SR proteins were also dispersed from nuclear speckles during the SRPK1 treatment, anti-Sm (Fig. 4Af and h) and anti-m⁷GpppG cap antibodies (data not shown) were used to detect snRNPs; disassembly of nuclear speckles was again observed. This result suggests that the localization of snRNPs to nuclear speckles may depend on the SR proteins, and SRPK1 may cause disassembly of the speckles, rather than just disassociation of SR proteins from them. Interestingly, a few Sm-positive foci remained in the nucleus after treatment with purified SRPK1 (Fig. 4Ah). This observation indicates that 'coiled bodies', distinct nuclear structures which contain snRNPs and nucleolar antigens³, may not be affected by this kinase. Double-labelling of cells with anti-Sm antibody (Fig. 4Aj and l) and with anti-p80 coilin (Fig. 4Ai and k) antibody, which is specific for coiled bodies²¹, showed that the unaffected foci were coiled bodies. SRPK1 is therefore selective for a defined nuclear structure, the nuclear speckles.

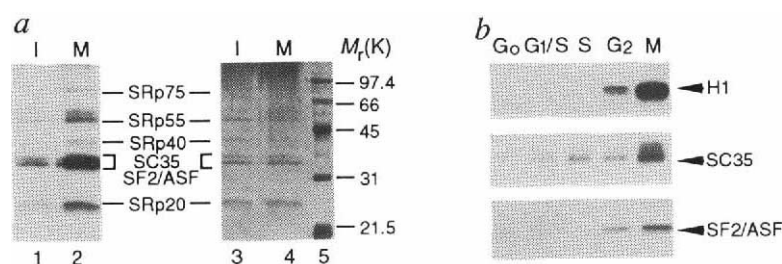
SR proteins are essential splicing factors that commit precursor mRNA to splicing and mediate spliceosome assembly²²; phosphorylation may therefore affect their activity in splicing. Splicing of human β -globin pre-mRNA was progressively inhibited by increasing doses of purified SRPK1 *in vitro* (Fig. 4B),

suggesting that SRPK1 could be a regulator of splicing in interphase cells, although precisely how is not clear. We cannot rule out the participation of other kinase substrates in the observed inhibition, but the simplest explanation is that an excess of SRPK1 causes hyperphosphorylation and/or prevents dephosphorylation of SR proteins, and that phosphorylation/dephosphorylation of SR proteins is required for splicing. Consistent with this, some SR proteins, including SC35 (ref. 1) and SF2/ASF²³ are detected as doublets in cell extracts, indicating that SR proteins in different phosphorylation states are present in cells. It is known that phosphatases are required for splicing but not for spliceosome assembly²⁴, and that incorporation of a non-hydrolysable homologue of ATP into the U1 70K protein by its associated kinase inhibits splicing²⁵, suggesting that dephosphorylation is necessary for splicing. Thus for the assembly of spliceosomes on pre-mRNAs, SR proteins may need to be phosphorylated, whereas for resolution of spliceosomes and recycling of splicing factors, these proteins are probably dephosphorylated.

In summary, we have identified and cloned a novel cell-cycle-regulated kinase, which is specific for the SR domain in splicing factors. We have demonstrated the function of SRPK1 in pre-mRNA splicing and in the cellular distribution of splicing factors during the cell cycle. The regulation of SRPK1 activity provides a potential target for post-transcriptional gene regulation in response to intracellular or external signals. □

FIG. 3 Cell-cycle regulation of SR protein phosphorylation *in vivo* and *in vitro* by SRPK1. a, Phosphorylation of SR proteins *in vivo* is cell-cycle-regulated. SR proteins as indicated were isolated from *in vivo* ³²P-labelled HeLa cells arrested at interphase (I) or metaphase (M), and silver-stained (lanes 3 and 4). The ³²P-labelled proteins in the stained gel were detected by autoradiography (lanes 1 and 2). Molecular markers (Bio-Rad) are shown in lane 5. Quantification of sliced bands showed that SR proteins were phosphorylated 3 to 5-fold more in M than in I phase cells. b, Activities of SRPK1 in extracts. Cell extracts made from HeLa cells arrested at different stages of the cell cycle as indicated at the top were assayed for kinase activity towards H1, SC35 and SF2/ASF (left). ³²P-labelled proteins were resolved by SDS-PAGE and detected by autoradiography. Quantification of sliced bands showed that the H1 kinase was about 5-fold in G2 and 20-fold in M higher than in G0, G1/S and S phase cells. SRPK1 assayed by both SC35 and SF2/ASF was slightly higher in G2, and 3–5-fold higher in M than in G0, G1/S, and S phase cells.

METHODS. Cells grown in three 150-mm culture dishes were arrested at G1/S or at M phase as described²⁷. After washing with phosphate-



free medium, cells were labelled for 3 h in phosphate-free medium plus 10% FCS, 10% DMEM and 0.25 mCi ml⁻¹ ³²P_i (NEN), then washed in PBS and SR proteins isolated according to ref. 13. SR proteins from interphase and mitotic cells were separated by SDS-PAGE, silver stained and autoradiographed. Cells were extracted at different stages of the cell cycle as described²⁷. H1 kinase assay was as for Fig. 1; to assay for SRPK1, we took the advantage of the tight binding of SRPK1 to phosphocellulose P11 resin, which allows other kinases to be washed away with high salt to reduce background. Bound SRPK1 was assayed using SC35 and SF2/ASF as substrates as described for Fig. 1.

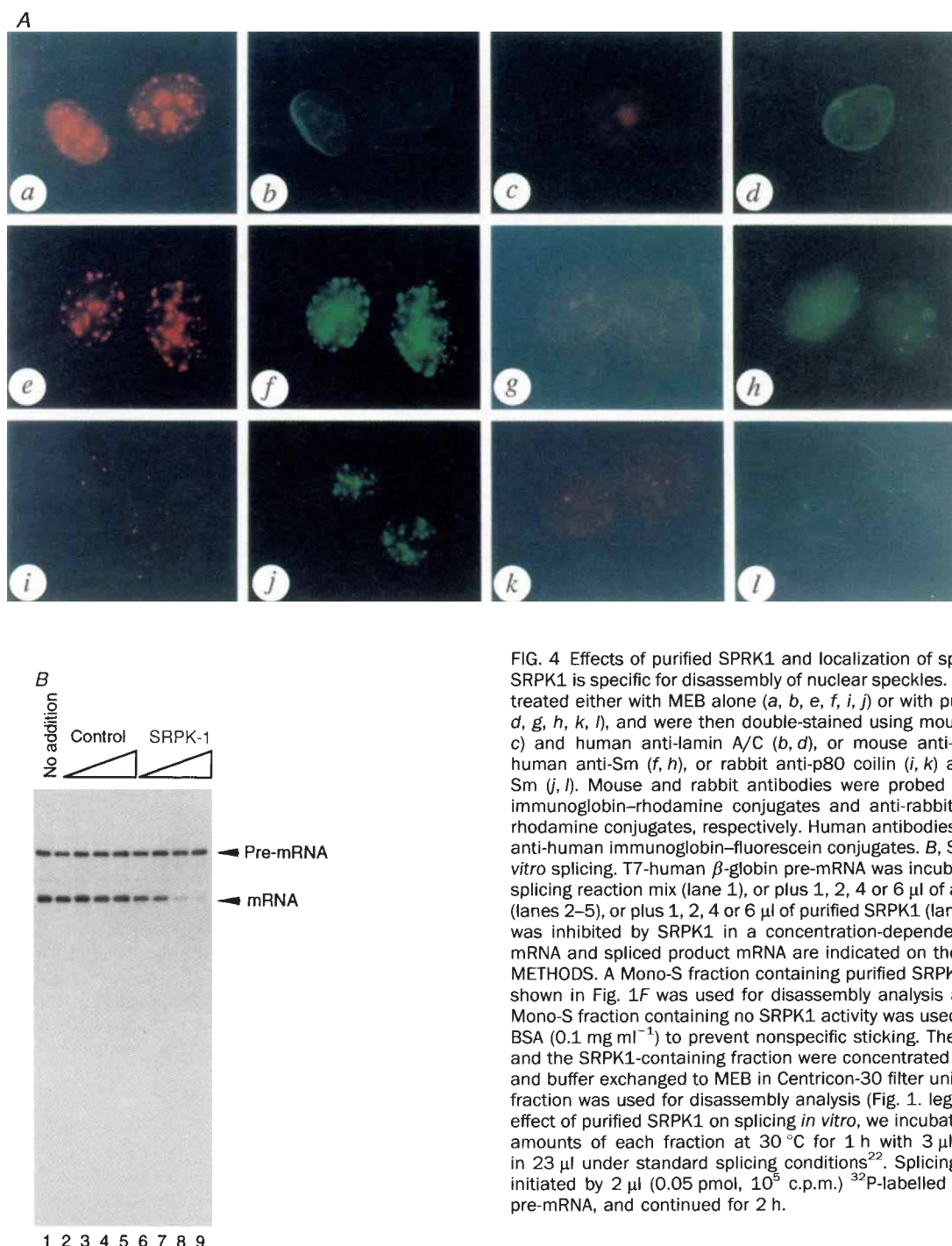


FIG. 4 Effects of purified SRPK1 and localization of splicing factors. **A**, SRPK1 is specific for disassembly of nuclear speckles. MG63 cells were treated either with MEB alone (**a**, **b**, **e**, **f**, **i**, **j**) or with purified SRPK1 (**c**, **d**, **g**, **h**, **k**, **l**), and were then double-stained using mouse anti-SC35 (**a**, **c**) and human anti-lamin A/C (**b**, **d**), or mouse anti-SC35 (**e**, **g**) and human anti-Sm (**f**, **h**), or rabbit anti-p80 coilin (**i**, **k**) and human anti-Sm (**j**, **l**). Mouse and rabbit antibodies were probed with anti-mouse immunoglobulin-rhodamine conjugates and anti-rabbit immunoglobulin-rhodamine conjugates, respectively. Human antibodies were probed by anti-human immunoglobulin-fluorescein conjugates. **B**, SRPK1 inhibits *in vitro* splicing. T7-human β -globin pre-mRNA was incubated in standard splicing reaction mix (lane 1), or plus 1, 2, 4 or 6 μ l of a control fraction (lanes 2–5), or plus 1, 2, 4 or 6 μ l of purified SRPK1 (lanes 6–9). Splicing was inhibited by SRPK1 in a concentration-dependent manner. Pre-mRNA and spliced product mRNA are indicated on the left.

METHODS. A Mono-S fraction containing purified SRPK1 ($2 \mu\text{g ml}^{-1}$) as shown in Fig. 1F was used for disassembly analysis and an adjacent Mono-S fraction containing no SRPK1 activity was used as control, with BSA (0.1 mg ml^{-1}) to prevent nonspecific sticking. The control fraction and the SRPK1-containing fraction were concentrated 5-fold, desalted, and buffer exchanged to MEB in Centricon-30 filter units; 20 μ l of each fraction was used for disassembly analysis (Fig. 1. legend). To test the effect of purified SRPK1 on splicing *in vitro*, we incubated the indicated amounts of each fraction at 30°C for 1 h with 3 μ l nuclear extract in 23 μ l under standard splicing conditions²². Splicing reactions were initiated by 2 μ l (0.05 pmol , 10^5 c.p.m.) ^{32}P -labelled human β -globin pre-mRNA, and continued for 2 h.

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Long PCR

Suzanne Cheng, Sheng-Yung Chang, Patti Gravitt and Richard Respass

As increasingly longer DNA targets are amplified reliably, new applications for PCR are becoming possible.

Use of the polymerase chain reaction (PCR) to amplify DNA sequences has been widespread in molecular genetics research, including genome mapping and sequencing studies¹⁻³. However, robust amplification of targets greater than ~5 kilobases (kb) has been difficult, limiting some applications of PCR. Recently, conditions were identified for effective amplification of longer DNA targets^{4,5}. The most successful protocols combined modifications to standard PCR buffers and thermal cycling profiles with a two-polymerase system to provide optimal levels of both processive polymerase activity and proofreading or 3'-to-5'-exonuclease activity. These conditions were used to amplify up to 22 kb from human genomic DNA, 42 kb from the bacteriophage lambda genome and, more recently, 16.3 kb of the 16.6-kb human mitochondrial genome^{5,6}. Here, we illustrate the application of these long PCR protocols to amplify introns of a newly identified gene and nearly entire viral genomes.

General method

Primer design. Successful primers will ultimately be determined empirically, but certain guidelines can be considered in choosing primer sequences. As with all PCR primers, the potential for secondary structure and dimer formation should be minimized. Typical primers for longer PCR amplifications have been 21–34-mers with balanced melting temperatures near 65–70 °C. Such primers permit the use of higher annealing temperatures to enhance reaction specificity. This can be critical, as the amplification of long targets will be compromised by preferential amplification of shorter nonspecific products. Gel-purification of primers is not essential, but may remove problematic impurities.

PCR conditions. Recombinant (r) *Tth* DNA Polymerase, XL (r*Tth* DNA polymerase from *Thermus thermophilus*⁷, Perkin-Elmer, Foster City, California with Vent DNA polymerase from *Thermococcus litoralis*⁸, New England Biolabs, Beverly, Massachusetts) was used for the work presented here, based upon conditions described previously⁵. A Tricine, pH 8.7 (at 25 °C) buffer provides a higher pH environment⁹ than that of the typical Tris-HCl, pH 8.3 PCR buffer, offering DNA strands greater protection against depurination and subsequent nicking during thermal cycling. Glycerol and dimethyl sulphoxide (DMSO) in the reaction buffer effectively lower melting and strand-separation temperatures (P. Landre and R. Watson, personal communication; refs 10,

11), thus facilitating denaturation of the template and enhancing specificity of the reaction. Glycerol may also enhance the thermal stability of the enzymes (although DMSO may be destabilizing), as has been observed with AmpliTaq (recombinant *Taq* DNA polymerase from *Thermus aquaticus*¹², Perkin-Elmer) DNA polymerase (P. L. and R. W., personal communication). Thermal cycling profiles varied with target lengths and primer sequences, but generally consisted of a Hot Start¹³ at 78–80 °C; initial denaturation at 94 °C for 1 min; 25–40 cycles of denaturation at 94 °C for 15 s, and annealing and extension steps at a fairly high temperature (for example, 60–68 °C) for 30–60 s per kilobase of target; and a final hold at 72 °C for 10 min. All amplifications were performed in GeneAmp PCR System 9600 thermal cyclers (Perkin-Elmer). Long PCR amplifications appear sensitive to thermal cycler characteristics; thus optimal protocols may differ among types of cyclers. As yields of longer PCR products may be comparable to yields from plasmid minipreps,

steps were taken to control product carryover contamination¹⁴.

Applications

Long PCR technology promises to facilitate many studies in molecular genetics. As examples, we describe amplifications of targets within the human *MSH2* (*hMSH2*) gene, the proviral HIV-1 genome, and the human papilloma virus type 16 (HPV 16) genome. **Human *MSH2* gene.** Hereditary non-polyposis colorectal cancer (HNPCC, also known as Lynch syndrome) is defined by early-onset colon cancer and other specific cancers within first-degree relatives of at least two generations, and is estimated to affect as many as 1 in 200 individuals in the western world¹⁵. The *hMSH2* gene is a human homologue of the bacterial *MutS* mismatch repair gene, and mutations within *hMSH2* have recently been identified in kindreds affected by chromosome 2-linked HNPCC^{16,17}. Detailed information about the prevalence and penetrance of mutations in *hMSH2* is essential for the development of

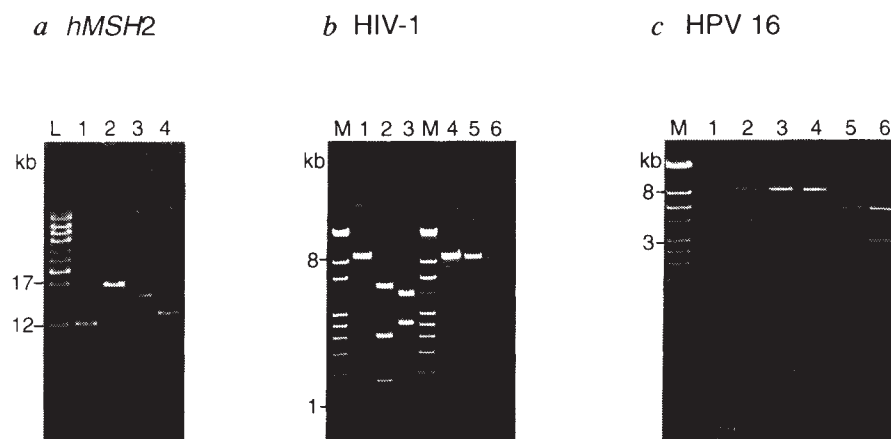


FIG. 1a, Amplification of segments within the *hMSH2* gene using primer pairs: exon 9/exon 12 (lane 1), exon 8/exon 9 (lane 2), exon 5/exon 7 (lane 3) and exon 1/exon 6 (lane 4). Targets were amplified from 100 ng genomic DNA in 100 µl, with 37–40 cycles at extension times of 12–17 min. Aliquots of 1–5 µl were electrophoresed on a 0.4% SeaKem Gold agarose gel (FMC BioProducts, Rockland, Maine) in Tris-acetate/EDTA. Lane 'L' contains High Molecular Weight DNA Marker from GIBCO-BRL (Gaithersburg, Maryland). b, Amplification of 8.3 kb from proviral HIV-1 plasmid (Z226; GenBank Accession No. M22639, National Center for Biotechnology Information, National Library of Medicine, the National Institutes of Health, Bethesda, Maryland) at 10⁶ copies per 100-µl reaction (lane 1). Product was verified by *ScaI* (lane 2) and *EcoRI* (lane 3) digests. Lanes 4–6 show titration of the plasmid: 10⁶, 10⁵, 10⁴ copies (27 cycles of amplification). Samples (for PCR samples, 10-µl aliquots) were electrophoresed on a 0.7% SeaKem GTG agarose gel (FMC BioProducts) in Tris-borate/EDTA. Lanes marked 'M' contain DNA Molecular Weight Marker IV from Boehringer Mannheim (Indianapolis, Indiana). c, Amplification of the HPV 16 genome from *BamHI*-digested HPV 16 plasmid at 10⁶ (lane 1), 500 (lane 2) and 1,000 (lane 3) copies per 100-µl reaction, after 35 cycles with 5-min total extension time. The genome was also successfully amplified from HPV 16-positive cervicovaginal lavage specimens (lane 4). Product from both purified plasmid (lane 5) and lavage sample (lane 6) verified by *HindIII* digestion. Samples (for PCR samples, 10-µl aliquots) were electrophoresed on a 0.6% SeaKem GTG agarose gel in Tris-borate/EDTA. Lane M is as in b. All gels were run in the presence of 0.5 µg ml⁻¹ ethidium bromide.

effective tests for HNPCC and related predispositions.

PCR amplification of *hMSH2* introns can expedite cloning and sequencing efforts, and thus identification of any clinically relevant mutations within the introns. Using primers located within the flanking *hMSH2* exons, introns of previously unknown size were amplified from total genomic DNA. For example, intron 8 appeared to be ~17 kb (Fig. 1a, lane 2). Replicate reactions and different primer pairs were used to avoid misidentification of large nonspecific products as the full-length target. Other regions spanning several exons and introns were also amplified (lanes 1, 3 and 4). Long PCR technology should accelerate determination of the genomic organization of *hMSH2* and other newly identified genes, and facilitate the association of specific genetic alterations with various diseases or predispositions.

Proviral HIV-1. Much of the difficulty in the development of drugs and vaccines effective against HIV-1 can be attributed to the rapid mutation of the viral genome, extensive heterogeneity of the viral population during different stages of infection, and difficulties in isolating infectious virus by cocultivation methods¹⁸. Such studies have been aided by PCR amplification of HIV half-genomes¹⁹, and could benefit further from reliable amplification of larger segments.

Figure 1b illustrates effective amplification of 8.3 kb of HIV-1 sequence from a plasmid containing the entire HIV-1 genome (lane 1), and verification of the products by restriction analyses (lanes 2 and 3). The current sensitivity is 10⁴ copies with 27 cycles of amplification (lane 6), and between 10 and 100 copies with 40 cycles (data not shown). Studies are underway to determine if full-length viral genomes can be amplified directly from clinical samples. Long PCR of the proviral HIV-1 genome will be useful for the analysis of viral heterogeneity and may provide insight into the design of effective drugs and vaccines.

HPV 16. Human papillomaviruses have a genotype-specific association with cervical²⁰ and other anogenital cancers²¹. Although epidemiological evidence suggests that these viruses are the primary cause of cervical cancer²², much of the natural history of HPV is unknown. PCR amplification of entire HPV genomes may overcome some of the limitations imposed by the inability to culture these viruses, and accelerate understanding of the biology of HPV.

HPV 16, for which the entire genome has been sequenced²³ and cloned²⁴, was selected for feasibility studies. Primers complementary to the recessed 3'-ends of the *Bam*HI-linearized genome were designed to regenerate the *Bam*HI site and incorporate new restriction endonuclease recognition sites and a 5'-terminal GGGG clamp for subsequent cloning. As shown in Figure 1c, the entire 7.9-kb genome was amplified from dilutions of HPV 16 plasmid (lanes 1–3) and from cervicovaginal lavage specimens us-

ing 5 µl of crude proteinase K/Laureth-12 digests²⁵ (lane 4). These PCR products were verified by eight restriction analyses; *Hinc*II digests are shown (lanes 5 and 6). Additional HPV 16-positive lavages are being examined to determine the sensitivity of long PCR in clinical specimens and its general applicability to these viruses. By enabling study of the entire HPV 16 genome, long PCR amplifications may allow more detailed characterization of HPV 16 variants²⁶ and further investigations of genotype-specific disease associations. Extension of this technology to all HPV species should facilitate their characterization and classification.

Extending the technology

An upper limit on the size of targets for PCR amplification has not been identified. Integrity of the template is one key parameter; longer targets can be amplified from DNA with fewer nicks, indicating the need for careful sample preparation (unpublished observation). Methods to increase reaction specificity and to improve primer design by minimizing secondary-site similarities will also be critical for successful longer amplifications. With a greater understanding of how to optimize critical parameters, routine amplification of genomic targets of 30–40 kb should be possible.

Long PCR can supplement cloning, or be a labour-saving alternative, for studying the molecular genetics of large genomic segments. We have illustrated here applications to the study of gene structure and amplification of viral genomes. The technology promises to reduce the expense of and accelerate studies including the Human Genome Project, and to encourage formulation of new studies not presently feasible. □

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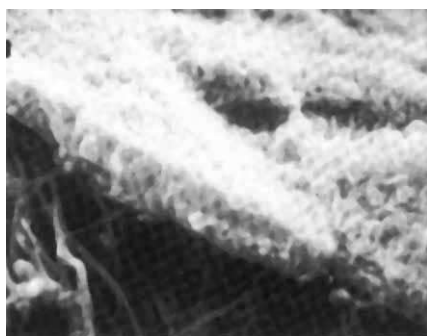
Tools of the research trade

New reagents to watch out for include 'live' cell nucleic acid stains, a yeast transformation kit, a chemiluminescent immunoblotting system and an expression system for the production of soluble proteins in *E. coli*.

FOR researchers in need of **nuclear acid stains that can penetrate live cells**, Molecular Probes announces six cell-permeant nucleic acid stains sold under the SYTO trademark (*Reader Service No. 101*). These stains, numbered SYTO 11 through to 16, differ from each other in cell permeability, degree of fluorescence enhancement with nucleic acid binding, excitation and emission spectra, and DNA/RNA specificity and binding affinity. They are compatible with either conventional broadband illumination sources such as xenon- or mercury-arc lamps, or monochromatic sources such as an argon-ion laser. The unbound dyes are said to have low intrinsic fluorescence, so stained cells can be observed directly after staining, without the need for additional preparation or washing steps. Stated applications include labelling cultured animal cells, plant cells, fungi and bacteria, and for measuring DNA or RNA concentration in solution.

Cell culture

Collaborative Biomedical Products/Becton Dickinson has developed a serum-free cell culture **system for the differentiation of intestinal epithelial cells in vitro** (*Reader Service No. 102*). The BioCoat intestinal epithelium differentiation environment integrates a serum-free medium supplemented with hormones, growth factors, defined



BioCoat intestinal epithelium differentiation environment.

nutrients and sodium butyrate, and BioCoat fibrillar collagen cell culture inserts (containing 1 µm microporous membranes with a deposition of large fibrils of rat tail collagen I). The system is designed to promote the formation of a differentiated enterocyte monolayer demonstrating barrier function within 2 to 3 days post seeding which, the company says, is significantly earlier than with more conventional methods. Stated applications include transport and absorption studies of drugs, nutrients and trace elements, as well as studies of infectious diseases of the intestine.

Ex-Cell PF CHO is a new **protein-free medium** from JRH Biosciences for the growth of Chinese hamster ovary cells (*Reader Service No. 103*). Because it contains no large molecules, the company says that the medium facilitates the isolation and purification of secreted proteins. It contains no animal-derived components and is supplied in liquid or powdered formulations.

Molecular means

BIO 101's **alkali-cation yeast transformation kit** provides a non-electrical method of transforming yeast cells with linear or circular plasmid DNA (*Reader Service No. 104*). Host cells are made competent through treatment with a lithium/caesium acetate mixture. Stated yields of greater than 10⁴ transformants per microgram of YEP24 plasmid

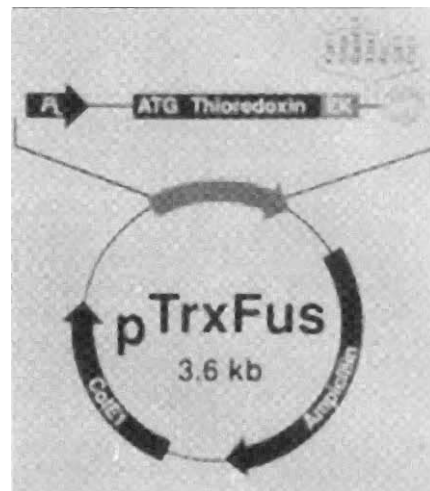


Yeast cell transformation kit.

DNA can be obtained. Each kit contains sufficient reagents for 25 1-ml preparations of competent yeast for 5–10 transformations per millilitre.

Western-Light Plus is the new **nonisotopic western blotting system** from Tropix designed to generate a high-intensity luminescent signal that is said to persist from hours to days (*Reader Service No. 105*). The tertiary detection system, consisting of primary antibody, biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate, permits film exposures using X-ray or instant film. Biotinylated molecular weight markers are included for concurrent detection with samples. The Western-Light Plus system is supplied with CSPD chemiluminescent substrate, Nitro-Block enhancer, blocking reagent, a choice of biotinylated secondary antibodies, streptavidin-alkaline phosphatase conjugate and biotinylated molecular weight markers optimized for luminescent detection.

Looking for an **expression system to produce soluble proteins in *Escherichia coli***? If so, Invitrogen might have the answer



Kit for soluble protein expression in *E. coli*.

with its new ThioFusion expression system designed to eliminate solubilization and time-consuming refolding schemes that are necessary when proteins form inclusion bodies (*Reader Service No. 106*). The company says that proteins expressed as N-terminal fusions with thioredoxin are often soluble—even at high levels of expression. In addition, the ThioFusion system provides two ways to purify recombinant protein. Each kit is supplied with expression and control vectors, strains of *Escherichia coli*, media components, sequencing primers and a manual.

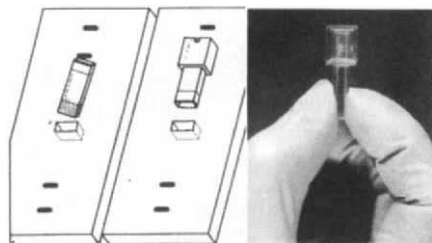
Biometra says that the GeneExScreen Primer Kit contains all the primers necessary to perform the complete **analysis of all the mRNA species present within a cell** at a given point in its cell cycle or state of differentiation, using differential display reverse transcription polymerase chain reaction (DDRT-PCR) amplification (*Reader Service No. 107*). The method is based on the assumption that every individual mRNA present in a cell, representing a mathematically estimated 15,000 active genes, could be reverse transcribed and amplified with a set of arbitrary primers. By using 12 downstream primers, 12 different cDNA populations are generated by reverse transcription. Each of these cDNA populations is amplified with a set of 26 upstream primers in 26 separate reactions. The products of the 312 individual reactions are analysed by electrophoresis, followed by autoradiography and scanning densitometry.

Founded in 1990, Monomer Sciences, primarily produces **speciality DNA and RNA intermediates** on a custom basis (*Reader Service No. 108*). In particular, the

company is a large-scale producer of DMT-N-protected nucleosides, succinic acids, derivatized CPGs, ribo 2' OMe protections, ribo 2' or 3' TBDMS protections, oligo (dT) cellulose and custom protections.

■ Isolation

Genecapsule is a handy new device from Geno Technology intended for the rapid recovery of DNA from gels (*Reader Service No. 109*). The device comes in two parts



DNA extraction device.

— the Gel-pick and the Gel-Trap — and uses the principle of electroelution to extract DNA from the gel. Extraction time depends on size and the electrophoresis conditions: it takes less than 60 s to recover 1,000 base pairs DNA, says the company. DNA recovered in this way is ready for ligation, restriction enzyme digestion, sequencing, amplification, random priming, nick translation and other enzymatic reactions. Recovery is said to be over 90 per cent in 10–30 μ l. Each kit is supplied with 54 Genecapsule units.

The three new Puragene DNA isolation kits from Gentra Systems are designed for the isolation of genomic DNA from whole blood, bone marrow, plant and animal tissues, cultured cells, bacteria and yeast (*Reader Service No. 110*). The kits are based



Puragene DNA isolation kits.

on a modified salt precipitation method, where DNA is typically prepared in 50 min for whole blood, to 2 h for tissues, says Gentra. Stated yields range from 35 μ g ml^{-1} for whole blood (7 million cells) to 0.5–10 μ g mg^{-1} of tissue. The average length of recovered DNA is 100 kb with A_{260}/A_{280} ratios of 1.7–2.0. The kits are complete (ex-

cept for alcohol) and do not use any toxic chemicals. DNA may be used for amplification, Southern blot, sequencing or other DNA analysis procedures. Three sizes of kit are offered; free trial-size kits are available on request.

■ Sequencing and synthesis

On the DNA sequencing front, Advanced Biotechnologies is offering two new kits for molecular biologists (*Reader Service No. 111*). The first is the Sequen AB kit for acquiring sequence information from a variety of templates using either radiolabelled or fluorescent primers or nucleotides. The second, Cyclase, contains all the components required for thermal cycle sequencing. ABI says this methodology eliminates the need for alkali denaturation and ethanol precipitation of double-stranded templates, and makes it possible to obtain sequence information from femtomole amounts of template.

The reagent tobacco acid pyrophosphatase (TAP) intended for RNA sequencing, mapping and labelling applications can now be obtained from Epicentre Technologies (*Reader Service No. 112*). It removes the cap (GpppG) structure from mRNA and other RNAs, leaving a 5'-phosphorylated terminus on the molecule. The resulting 5'-phosphorylated RNA may be used in several applications. For example, the company says intramolecular ligation of a 5'-phosphorylated viral genomic RNA has been used to determine the nucleic acid sequence of the 3' terminus. The 5'-termini of mRNAs may be mapped by ligation of oligoribonucleotides to TAP-treated RNAs followed by amplification. 'Decapped' RNAs may also be 5'-end labelled for sequencing or for use as hybridization probes.

AnaSpec's new 2-chlorotriptyl resin is designed to eliminate some of the problems associated with peptide synthesis using Fmoc chemistry (*Reader Service No. 113*). Aside from suppressing the formation of diketopiperazines, the company says that additional advantages to using its resin include racemization-free esterification for loading His, Cys and Phe on the resin, complete cleavage of C-terminal Trp and Met peptides, and no homoserine formation of C-terminal Met.

■ Immunoassays

A TiterZyme mouse interleukin-4 'sandwich' enzyme immunoassay kit for the quantitative determination of mouse IL-4 levels in serum or cell culture supernatant is now available from PerSeptive Diagnostics (*Reader Service No. 114*). Detachable well-strips, coated with mouse IL-4, are designed to provide flexibility and consistency for multiple runs. Prediluted, lyophilized standards are included. The company says that only 50 μ l of sample is required and the assay can be completed in less than 3 h. The stated sensitivity of the enzyme immunoassay

is 2.25 pg ml^{-1} in buffer; less than 1 pg ml^{-1} in serum diluent. Sufficient reagents are included for 96 tests, including standard curves.

Biolab offers six extractable nuclear antigen, colour-controlled ELISA kits based on affinity purified native SSA, SSB, Sm, Sm-RNP, Scl-70, Jo-1 antigens (*Reader Service No. 115*). Colour-coded reagents, together with a convenient colour change



Colour-controlled ENA ELISAs.

feature (marking each significant procedural step), are designed to reduce the likelihood of handling errors and the need for repeat testing. Tests can be run either qualitatively with a ready-to-use cut-off calibrator or quantitatively with four to six ready-to-use standard curve calibrators.

Moss introduces a soluble TMB (3,3',5,5'-Tetramethylbenzidine) substrate for ELISAs that can be 'stopped' for an hour or more (*Reader Service No. 116*). This new single-component liquid substrate for ELISAs is stable at room temperatures for up to 18



TMB-ELISA-Stop.

months, says Moss. It provides a blue, soluble end product that may be quantified at 650 nm. Addition of acid converts the blue colour to yellow, which can be read at 450 nm. Other horseradish peroxidase substrates available from Moss include TMB-membrane, DAB (3,3'-Diaminobenzidine), ABTS (2,2'-Azino-bis-[3-ethylbenzthiazoline-6-sulphonic acid]) and AEC (3-Amino-9-ethylcarbazole); alkaline phosphatase substrates include BCIP/NBT (5-Bromo-4-chloro-3-indoyl phosphate/Nitroblue Tetrazolium), p-NPP (p-Nitrophenyl phosphate), BCIP/TNBT (BCIP/Tetranitroblue Tetrazolium), BCIP/INT (BCIP/p-Iodi-

PRODUCT REVIEW

nitroterazolum), new Fuchsin and Fast Red Violet.

■ Antibodies

Rockland Immunochemicals is offering **antibodies to specific components of the highly conserved REL/NFκB family** of transcriptional regulators (*Reader Service No. 117*). Included among these are: anti-p50 (rabbit), anti-p65 (rabbit), anti-cRel (rabbit), anti-IκB (rabbit), each of which costs US\$150, and a US\$550 anti-NFκB kit, which includes 100 µl each of the above

antisera, 1 unit of each control peptide and 2 mg of peroxidase-conjugated, affinity-purified anti-rabbit IgG (goat). Antisera against NFκB subunits p50, p65 and cRel and the regulator IκB are available in the form of buffered antiserum in 0.1-ml aliquots preserved with sodium azide. Stated applications include immunoprecipitation, western blotting, gel-supershift assays and other immunological techniques.

Chemicon has introduced a line of **monoclonal and polyclonal antibodies to peripherin** — a member of the intermediate filament family and closely related to vimentin, desmin and glial fibrillary acidic protein (*Reader Service No. 118*). The antibodies are designed to work on western blots and frozen sections of tissues of all mammals tested to date, including human, rat and mouse. They also work on



Antibodies to peripherin.

several cell types in tissue culture, including rat hippocampal neurons, sensory neurons and PC12 cells, says Chemicon.

The monoclonal antibody, MIB-1, is now available from AMAC for the **detection of the Ki-67 antigen** in routinely fixed, paraffin-embedded tissues after an antigen-recovery step (*Reader Service No. 119*). It may also be used on frozen sections and cytological samples. MIB-1 reacts with the Ki-67 nuclear antigen (345- and 395-kilodalton double band in western blot analysis of proliferating cells) associated with cell proliferation and found throughout the cell cycle (G_1 , S , G_2 and M phases), and is absent in resting (G_0) cells. It can be used in the study of cell proliferation in neoplastic cell populations and stimulated lymphocytes.

Dako's new **monoclonal anti-human neuroblastoma antibody** is intended for use on routinely processed, formalin fixed, paraffin-embedded tissue sections (*Reader Service No. 120*). The antibody is said to react with a 57-kilodalton, fixation-resistant epitope and can be used to differentiate neuroblastoma from neural or haematopoietic tissues. Other tumour-associated antigen antibodies from Dako include Ewing's sarcoma marker, HMB45 and thrombomodulin. Additionally, there is a line of alkaline phosphatase and peroxidase-based immunohistochemical visualization systems.

Cymbus Bioscience has a new **anti-p53 antibody** that is intended for the detection of p53 overexpression in clinical specimens and for investigations of p53 tumour suppressor gene function and abnormalities in cancer (*Reader Service No. 121*). The company says that its antibody has been used in ELISAs and to stain western blots and frozen tissues fixed with ethanol and formol saline; cross-reactivity with other proteins has not been

detected. It is available as a 1-ml supernatant containing 10 mM sodium azide.

■ Miscellaneous reagents

Preparing buffers should be a snap with the new **colour-coded pH buffers** from Appleton Woods (*Reader Service No. 122*). The colour-coded capsules are designed for quick and easy identification of the prepared solution: an orange capsule indicates an acidic solution (pH 4), green indicates neutral (pH 7), and blue denotes an alkaline solution (pH 9 or pH 11). After emptying the capsule and dissolving its contents, the case is dropped into the buffer solution, so aiding identification.

Protein phosphatase 1 (PP1γ-isoform) and protein phosphatase 2B (PP2B, calcineurin) are two types of **protein serine/threonine phosphatases** in eukaryotic cells. Both enzymes are now available from Boehringer Mannheim in a purified form without contaminating kinases and proteases (*Reader Service No. 123*). PP1 is a recombinant enzyme expressed in *Escherichia coli* with a molecular weight of 37 kilodaltons. It is said to exhibit comparable activities towards phosphorylase, phosphorylase kinase and myosin, and is activated 2–3 fold by $MnCl_2$. PP2B, on the other hand, is isolated from bovine brain. It binds to calmodulin in the presence of Ca^{2+} (by which it is activated) and shows a broad substrate specificity, dephosphorylating proteins that have been phosphorylated in most cases by cAMP- and/or calmodulin-dependent kinases.

Isolated from *Thermus brokianus*, Amresco's new Thermalase *Tbr* **thermostable DNA polymerase** provides molecular biologists with an alternative to *Taq* DNA polymerase (*Reader Service No. 124*). The enzyme is said to offer high fidelity and high thermostability: at 96 °C, the enzyme has a stated half life of nearly 2.5 h. The company says it can be used to extend primers up to 6,000 base pairs.



Thermostable polymerase.

Over 1,000 products, including catalysts, initiators and cross-linking monomers can be found in the 1994 **catalogue of polymers and monomers** from Polysciences (*Reader Service No. 125*). Also featured is a selection of Polybead polystyrene microparticles suitable for calibration work or filter challenges. Research as well as bulk quantities and custom synthesis are offered. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

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The use of temporary laboratory scientists

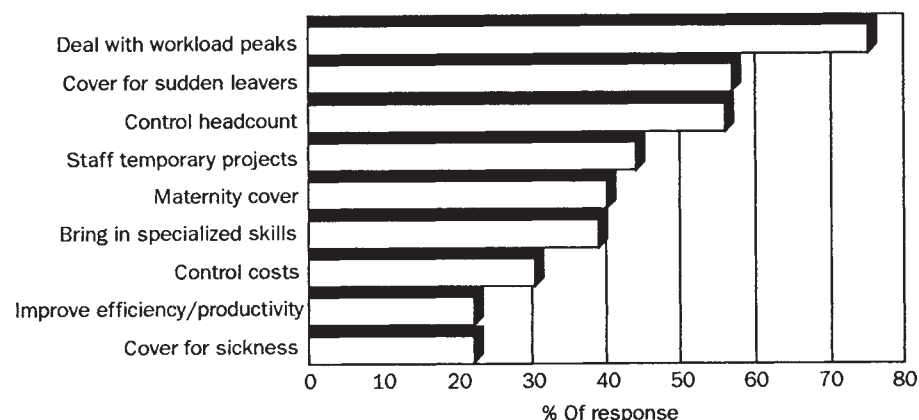
Ben Blackden

A comparison of trends and costs between two European Union countries, the United Kingdom and France, and the impact that regulation can have on employment opportunities.

SCIENTIFIC employers often face difficult situations that have a damaging effect on the efficiency of their laboratories and so on their organizations as a whole. Workload peaks, bottlenecks, cover for leavers, lack of specialist skills and slippages on contractual work all result in a cry for "Help!"

Help comes in the form of temporary scientists and technicians, carefully matched to bring exactly the right skills and experience to the laboratory for just as long as they are needed. Because specialized employment businesses are able to find the right needle in the haystack of scientific skills, this new breed of scientific employee is much in demand.

The supply of skilled scientists for temporary assignments is the role of the specialist employment business. Even major scientific employers who receive plenty of unsolicited job applications know how hard it is to find a scientist with the right skills at the right time; a specialist employment business is equipped with a dedicated database to provide the right candidates for the job. A laboratory or human resource manager will take considerable comfort when speaking to consultants who talk the same language and who understand what is needed. To be asked "Yes, you need skills in atomic absorption spectroscopy, but is that flame or graphite furnace?" really provides a degree of confidence that even the most demanding of scientific job specifications will be understood. This is not the busi-



How scientific employers have responded to surveys on the question of when they have most needed temporary help.

ness of the high street 'temp agency', which is simply not specialized enough.

The two figures here represent replies from more than 600 scientific employers in the United Kingdom who employed more than 1,500 temporary workers in 1993. Of these temporary workers, 30 per cent were transferred to permanent jobs at the end of their assignments. The temporary help industry — in this case a scientific employment business — can therefore make a marked contribution to the creation of employment opportunities. The 'temp-to-perm' statistics are particularly gratifying for the employees in what has been a grim period for permanent jobs. They also get a good rate of pay and, for the existing permanent workforce, an extra degree of security and protection from

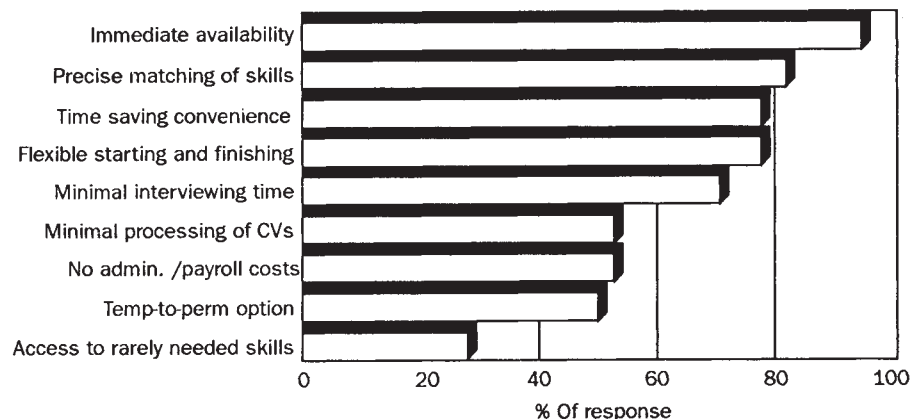
what might be the alternative 'hire and fire' approach.

There are few rules and regulations controlling or limiting the choices for employers; such regulations as do exist cover the proper protection of temporary workers in respect of health and safety issues, for example. There are (at present) some innocuous regulations concerning employment agency licensing. By and large, market forces dictate demand for temporary scientific workers; the professionalism and technical understanding of a scientific employment business can help a company to ensure the supply.

Within the European Union (EU) this freedom of operation applies not only in the United Kingdom but also in Denmark (where all restrictions regarding *travail intérimaire* were lifted in 1990) and the Republic of Ireland. In these countries, where market forces are allowed to apply, the relative lack of regulation is balanced by the application of general principles of labour law.

At the other end of the spectrum, however, there are three members of the EU — Italy, Spain and Greece — where employment businesses and temporary work are actually illegal (see *Temporary work and Labour law of the European Union and Member States*, edited by R. Blanpain, Kluwer Law & Taxation Publishers, 1993). No wonder the community as a whole has such difficulty with the social provisions of the Maastricht Treaty.

Of the rest of the continental members



The importance attached by scientific employers to the benefits of temporary technical staff through a specialist employment business.

A summary of some aspects of temporary working regulated in France

When temporary workers may be used

Duration of assignments

Extension of assignments

Premature termination of assignments

Consultation with Comité d'Entreprise

Employment businesses

"

Reporting to government

Contractual agreements

Rights & benefits for temporary workers,

only 3 legally permitted reasons

restriction including 18 month maximum

one only and subject to 18 month total

if no new assignment, wages to be paid in full

mandatory in certain circumstances

proof of financial security

cannot act for permanent jobs

detailed monthly reports

specific wording & contents

wage level protection; holiday 10%;

insecurity allowance 10%

of the EU, Belgium, Germany, France, the Netherlands and Portugal all fall between these extremes; between temporary work being illegal and a relatively unregulated temporary help industry. Specific legislations deal with temporary work: as Roger Blanpain puts it: "sometimes laying down very detailed rules to clearly define rights and obligations of the different parties involved, namely the temporary work business, the end-user and the temporary worker".

In his introduction to the French section of *Temporary Work and Labour Law*, Jacques Rojet (University of Paris) refers to "the difficulty of the balancing act demanded not to deprive enterprises of a much needed source of flexibility in manpower management . . . while avoiding the abuses in some companies . . . detrimental to the employee". He also points out that the contract of indeterminate duration (permanent employment) has been the rule in French labour law and all other contracts have been defined by exception. Rojet continues: "an added problem is that temporary work, in its strict definition, includes a tripartite relationship. French law is ill-fitted to such relationships. For instance it ignores the trust relationship familiar to Anglo-Saxon law".

One must accept that laws in any country stem from that country's historic perspectives, employment traditions and legal systems. There is no 'right' or 'wrong' and certainly no Anglo-Saxon sniping at Gallic ways intended here.

Some aspects of French law might well have applicability in the United Kingdom, just as there are perhaps aspects of regulation in France that have served their useful purpose and whose removal would benefit employers, employees and, above all, employment in France.

The financial guarantee. Every employment business in France must provide the government and its clients annually with proof of its financial security. This must be provided by a bank or insurance company. For new businesses it can take the form of a substantial cash deposit, the current minimum is about £45,000. This *attestation de garantie* protects the wages of the temporary workers in the event of the insolvency of the employment business. In the United Kingdom, when an employment business goes bust (as happened to a

medium sized company in the South of England last year) all its temporary workers are left without pay and little prospect of getting what they are owed, save for the goodwill of the user-employer. A financial guarantee for employment businesses could usefully be introduced in the United Kingdom (and is in sharp contrast with the 'red-tape bonfire' proposed by Mr Michael Heseltine, the president of the Board of Trade, in which the UK employment agency licensing regulations are to be abolished. These are innocuous but should be strengthened, not abandoned.)

For an industry with low barriers to entry and with some operators in need of more responsible management, such regulations could be a welcome addition, to prevent the operation of 'cowboys' as well as providing additional security to temporary workers.

One aspect of 'control' in France that is not so welcome is that an employment business can only provide temporary workers, it cannot, for example, also supply permanent workers. This law originates from the needs to stop unscrupulous agencies 'converting' permanent jobs into temporary ones. In the United Kingdom, a specialist employment business is able to use its technical knowledge and its specialized database of candidates to provide clients with temporary workers and with permanent workers as well as the 'temp-to-perm' transfer of temporaries. The latter (some 30 per cent of temporary assignments) not only helps the employer to avoid hiring mistakes, it also helps the employee to make the right career choice. In France, some relaxation of this regulation would, we believe, be very beneficial all round.

Rights of workers The main thrust of French law is to give temporary workers broadly the same rights and benefits as permanent employees. The rate of pay must be the rate for the job (and rightly so); in addition, 10 per cent of total earnings is paid for holidays and a further 10 per cent as an insecurity allowance at the end of each assignment. From the temporary worker's point of view, these are valued benefits and it would be wrong and impractical to suggest any diminution of them. The result, however, is that a temporary worker, usually supplied through a specialist employment business, is more expensive than a permanent em-

ployee. The extra is mainly, but not exclusively, the employment businesses' operating margin. In Britain, on the other hand, it is generally accepted that temporary workers supplied through a third party will represent a cost to the end user equivalent to that of a permanent employee.

Inevitably, the cost of a product or service has a bearing upon the extent to which it is used. The fact that temporary workers in the United Kingdom are not more expensive than their permanent counterparts provides more temporary work opportunities. In France it is a matter of balance between a high level of 'rights and benefits' for the temporary worker and keeping the cost to employers at a level that will attract greater use of those who would otherwise be unemployed. The transfer from *intérim* (temporary status) to *contrat à durée déterminée* (fixed-term contract) is subject to another regulatory anachronism in which a compulsory period of unemployment is legally required between the two.

It is in the general area of restrictive controls that change in France might be beneficial as well as practical. In the United Kingdom (and the United States) scientific employers have experienced how the use of skilled scientists and technicians on temporary assignments has been good management and employment practice giving improved operating efficiency, protection for core staff as well as employment opportunities and good rates of pay for temporary workers. The flexibility to offer such assignments, to extend (or terminate) them and to operate in an open and unrestricted way is very important for businesses generally, particularly as Europe as a whole faces ever stiffer competition from manufacturing and research capacity in the developing world.

The use of temporary workers — the flexible workforce — is on the increase and is not exploitative; an employer using temporary staff does not do so at the expense of permanent staff. More and more workers, at all levels and with all types of skill are actively looking for temporary work. Good and professional employment businesses provide the conduit for them to end-user employers.

Our experience shows that as specialist employment businesses actively marketing our services and believing absolutely in the value these bring to clients and candidates alike, we are adding to the number of employment opportunities, but in France, with a little less regulation, we could be doing so much more. □

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